Dissecting the non-canonical functions of p53 through novel target identification and p53 acetylation

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ABSTRACT

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It is well established that the p53 tumor suppressor plays a crucial role in controlling cell proliferation and apoptosis upon various types of stress. There is increasing evidence showing that p53 is also critically involved in various non-canonical pathways, including metabolism, autophagy, senescence and aging. Through a ChIP-on-chip screen, we identified a novel p53 metabolic target, pantothenate kinase-1 (*PANK1*). PanK1 catalyzes the rate-limiting step for CoA synthesis, and therefore, controls intracellular CoA content; *Pank1* knockout mice exhibit defect in β-oxidation and gluconeogenesis in the liver after starvation due to insufficient CoA levels. We demonstrated that *PANK1* gene is a direct transcriptional target of p53. Although DNA damage-induced p53 upregulates PanK1 expression, depletion of PanK1 expression does not affect p53-dependent growth arrest or apoptosis. Interestingly, upon glucose starvation, PanK1 expression is significantly reduced in HCT116 p53 (-/-) but not in HCT116 p53 (+/+) cells, suggesting that p53 is required to maintain PanK1 expression

under metabolic stress conditions. Moreover, by using p53-mutant mice, we observed that PanK activity and CoA levels are lower in livers of p53-null mice than that of wild-type mice upon starvation. Similar to the case in *Pank1* knockout mice, β-oxidation and gluconeogenesis are impaired in p53-null mice. Together, our findings show that p53 is critical in regulating energy homeostasis through transcriptional control of *PANK1*.

Our study on PANK1 led us to the question of how p53 can differentially regulate a diverse array of downstream targets in a context-dependent manner. Studies have shown that p53 acetylation at K120 and K164 lysine residues contribute to p53-mediated apoptosis and growth arrest functions, which was further supported by the 3KR mouse model (K117/161/162R) that mirrors the K120/164R mutations in human p53. These studies also suggest that a potentially large number of p53 targets can still be regulated by p53 in the absence of K120/164 acetylation (K117/161/162R in mouse). To investigate whether additional modifications of p53 can further contribute to promoter-specific transactivation, we conducted a screen using mass spectrometry and identified a novel acetylation site at K101. Our data demonstrated that K101 in human p53, as well as the homologous K98 lysine residue in mouse p53, can be acetylated by acetyltransferase CBP. Acetylation at this novel site does not contribute to p53 stability or DNA-binding capabilities. Ablation of K98 acetylation in mouse p53 alone does not

affect the transcriptional activity of p53. However, simultaneous loss of K98 acetylation with the previously characterized K117/161/162 acetylations (4KR98 p53) significantly abrogates p53-mediated activation of TIGAR and MDM2 genes.

The 3KR mouse model, although cannot elicit canonical p53-mediated apoptotic and cell cycle arrest responses, still retains the ability to suppress tumor formation. We, therefore, investigated whether other non-canonical targets of p53 could potentially mediate tumor suppression. By RNA-seq profiling of gene expression in cells expressing 3KR p53, we identified TNFRSF14 (tumor necrosis factor receptor superfamily, member 14) as a novel p53 target. The TNFRSF14 receptor has been shown to be frequently mutated in follicular lymphoma and diffuse large B cell lymphoma, and stimulation by its ligand LIGHT leads to cell death in many cancer cells. We report that TNFRSF14 is a novel p53 target that can be activated by 3KR p53. Interestingly, transactivation of TNFRSF14 is defective by 4KR98 p53. Furthermore, LIGHT ligand stimulates cell death in TNFRSF14-expressing cells and cells expressing 3KR p53, but not those expressing 4KR98 p53.

Altogether, our findings in these studies underscore the extensive scope of p53 functions and provide new insights into the versatility of non-canonical pathways. Not only does p53 mediate tumor suppression through both canonical and non-canonical

downstream effectors, p53 can also contribute to cellular homeostasis and energy balance.

Table of Contents

List of Figures

Acknowledgements

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1. General Introduction

1.1. p53 tumor suppressor

The p53 protein is a tumor suppressor that is found to be mutated in more than 50% of sporadic tumors, and is one of the most important tumor suppressors that prevents malignant transformation in mammalian cells [1-4]. The primary functions of p53 are mediated by its role as a transcription factor that binds sequence-specific DNA on its target promoters, and subsequently activates or represses target gene expression [5]. The importance of p53 transcriptional activity is highlighted by the observation that most tumor-prone p53 mutations occur within the central core domain responsible for DNA binding [2]. Individuals with Li-Fraumeni syndromes carry germline p53 mutations that confer high familial risk of cancer [2,6]. Deletion of p53 does not cause developmental defects, but results in a significantly shortened latency of cancer onset [7].

The p53 protein forms a homotetramer that recognizes p53 response element at target promoter containing two decamers with the following consensus sequence: PuPuPuC(A/T)|(A/T)GPyPyPy (Pu – purine; Py – pyrimidine) [8]. Each p53 subunit contains an N-terminal transactivation domain, a DNA-binding core domain, a tetramerization domain, and a C-terminal regulatory domain. The core domain binds to the consensus p53 sequence on the target promoter, while the transactivation domain recruits transcriptional machinery and co-activators to activate transcription. The

tetramerization domain allows four p53 subunits to come together as a tetramer, and the C-terminal domain is heavily regulated by post-translational modifications, which in turn modulate p53 transcriptional activity [9-12].

Classically, p53 functions as a master regulator that controls the expression of downstream targets that are responsible for cell fate control. In response to cellular stresses such as hypoxia, genotoxic and oncogenic stress, p53 activity and level is increased to induce growth arrest, senescence or apoptosis [13,14]. Under mild stress, p53 induces cell cycle arrest to allow cells to repair damage or recover from stress before resuming normal replication. Alternatively, upon severe stress that causes irreparable damage, p53 either triggers senescence, an irreversible cell cycle arrest, or activates a number of pro-apoptotic genes to terminate the cell, and thus, preventing potential oncogenic mutations to prevail [3].

Growth arrest function of p53 is predominantly carried out by the upregulation of p21 (WAF1) expression, as well as that of GADD45 and 14-3-3σ [15-17]. The p21 protein binds directly to and inhibits the activity of several cyclin-dependent kinase complexes (CDK1/2/4/6) that promotes cell cycle progression, and thus, halts cell cycle at G1 and S phases [15]. On the other hand, GADD45 and 14-3-3σ contribute to G2 arrest through inhibition of cyclin B/cdc2 [16,17].

Mediators of p53-dependent apoptosis include members of the Bcl-2 family of proteins - PUMA, BAX, and NOXA. The p53 upregulated modulator of apoptosis (PUMA) protein interacts and sequesters anti-apoptotic Bcl-2 family members, allowing pro-apoptotic proteins to initiate apoptosis [18,19]. One such protein is the Bcl-2-associated X (BAX) protein, which upon activation forms oligomeric pores on the outer membrane of mitochondria that result in the release of cytochrome C, leading to caspase-dependent apoptosis [20,21]. Similar to PUMA, NOXA undergoes BH3 motif-dependent localization to the mitochondria and interacts with anti-apoptotic Bcl-2 family members that result in subsequent caspase activation [22,23].

1.2. Non-canonical functions of p53

Recently, the roles of p53 have expanded beyond the canonical functions of apoptosis and cell growth arrest, and now include cellular processes such as metabolism, autophagy, senescence and aging [3,4]. While apoptotic and growth arrest functions of p53 has long been thought to mediate p53-dependent tumor suppression, emerging evidence suggest that non-canonical functions of p53 may also play critical roles in this process (**Figure 1.1**).

Figure 1.1

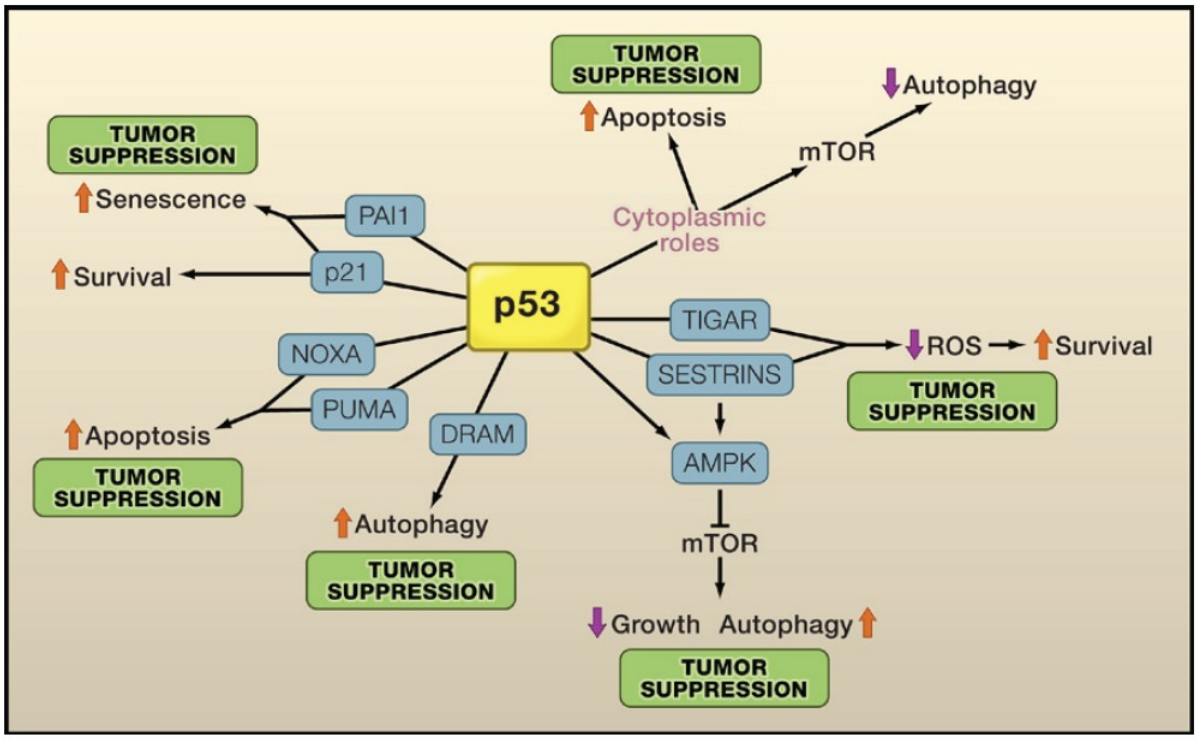

Figure 1.1. Tumor suppression by p53 is mediated through multiple functional branches. Aside from the canonical apoptotic and growth arrest functions, p53 can confer tumor suppression via regulation on senescence, autophagy, and metabolism. Most of these functions are carried out by downstream targets that are transcriptionally regulated by p53 (some examples shown in the blue boxes). However, p53 also assumes cytoplasmic roles in regulating autophagy and cell death that is independent of its transcriptional activity. (Figure reproduced from Vousden et al, Cell 2009)

1.2.1. Metabolism and oxidative balance

Not until recent discoveries that link p53 to cellular metabolism, tumor suppressing functions of p53 have long been attributed to its ability to regulate cell cycle through apoptosis, growth arrest, and senescence [24,25]. However, as tumorigenesis is increasingly viewed as a process that involves complex metabolic perturbations, the role of p53 in suppressing tumor formation is also increasingly seen as a metabolic one (**Figure 1.2**).

Cancer cells acquire numerous metabolic alterations to enable rapid growth and proliferation, including aerobic glycolysis (the Warburg effect) and enhanced biosynthesis of macromolecules [26,27]. Aerobic glycolysis allows cancer cells to rapidly generate ATP at the cost of efficiency while providing intermediates for de novo synthesis and oxidative balance. Processes such as aerobic glycolysis and glutaminolysis promote biosynthesis of protein, lipids and nucleic acids, which promote cancer cells to grow and proliferate more rapidly, and in some cases, confer survival advantages of tumor cells [28,29]. Reversing the Warburg effect compromises tumorigenicity and survival of cancer cells, while inhibiting glutaminolysis hinders oncogenic transformation of normal cells [30-32].

Interestingly, many p53 metabolic targets counteract the effects of the metabolic

Figure 1.2

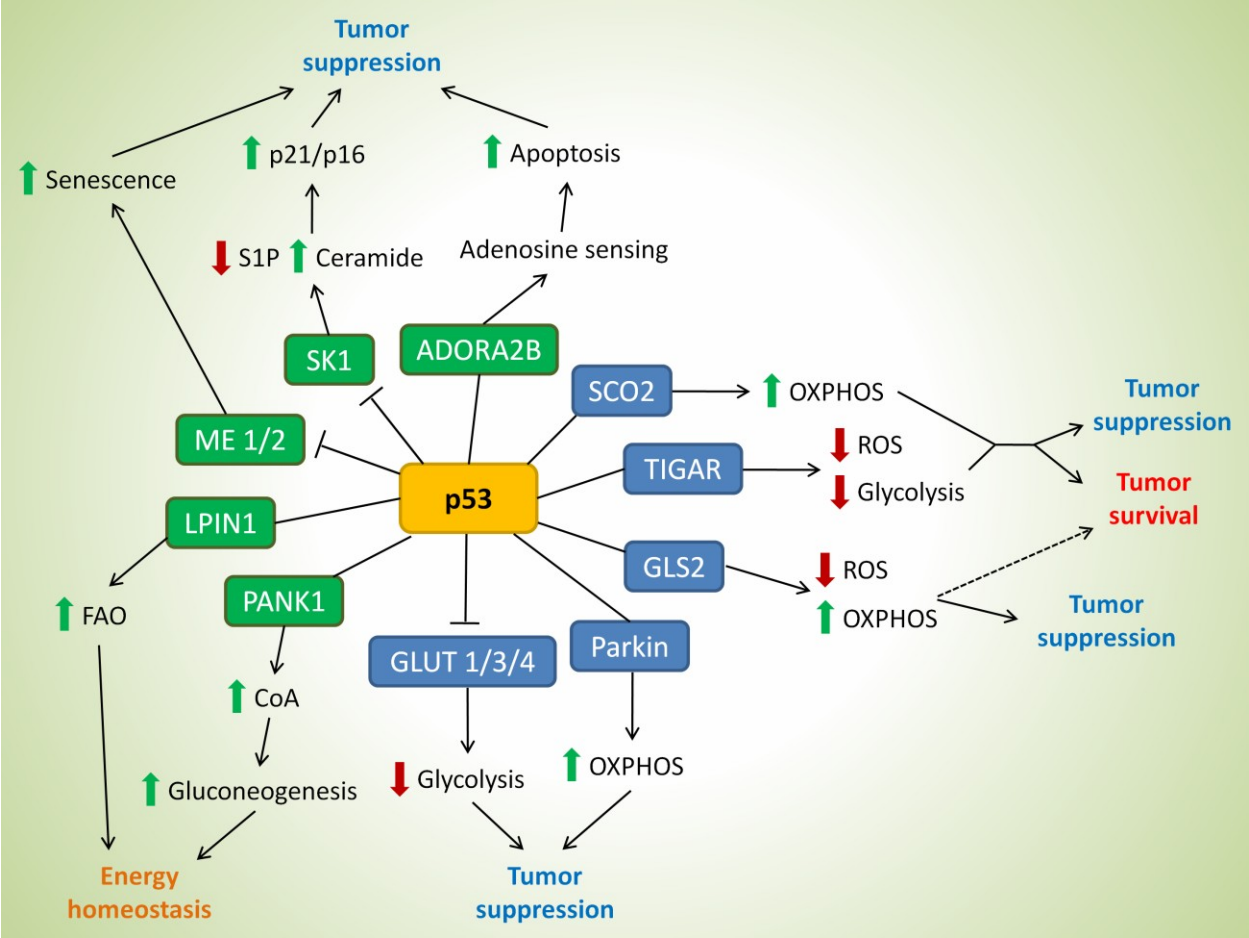

Figure 1.2. p53 exerts a diverse array of metabolic functions. A broad spectrum of metabolic targets are regulated by p53, leading to various functional outcomes in tumor and normal physiology. Genes in blue boxes are previously-identified p53 targets, while those in green boxes are most recently discovered. Dashed line in the GLS2 axis illustrates the potential of this target in enhancing tumor survival based on its function. *Abbr. OXPHOS, oxidative phosphorylation; S1P, sphingosine-1-phosphate; FAO, fatty acid oxidation; CoA, coenzyme A.*

alterations observed in tumor cells, which include inhibition of aerobic glycolysis, upregulation of mitochondrial OXPHOS, and promotion of fatty acid oxidation [24]. Additionally, several of these targets aim to increase antioxidant production and to lower intracellular reactive oxygen species (ROS), a protective mechanism to thwart ROS-induced DNA damage and subsequent malignant transformation [33,34]. As a tumor suppressor, p53 functions to bolster mitochondrial oxidative phosphorylation (through activating targets such as *SCO2*/*GLS2*/Parkin) and inhibits aerobic glycolysis (via expression of *TIGAR* and repression of *GLUT1/3/4*), which antagonize the predominant utilization of glycolysis in cancer cells [35-41].

Synthesis of cytochrome c oxidase (SCO2) regulates the cytochrome C oxidase complex of the electron transport chain and increases mitochondrial respiration [35]. Depletion of p53 resulted in the decrease in SCO2 expression, which led to a decrease in mitochondrial respiration and oxygen consumption. One isoform of glutaminase (GLS2), also a p53 target, converts glutamine to glutamate, which can either enter the TCA cycle or be used as a substrate for glutathione synthesis [36,37]. Functionally, expression of GLS2 contributes to mitochondrial respiration through increasing α-ketogluterate flux into the TCA cycle, while also lowering ROS levels through increasing antioxidant glutathione production. Expression of Parkin also promotes

mitochondrial oxidative phosphorylation by increasing the cellular level of acetyl-CoA through the upregulation of pyruvate dehydrogenase E1α1 [38].

Regulation on glycolysis by p53 include TP53-induced glycolysis and apoptosis regulator (TIGAR), which is an enzyme that converts fructose-2,6-bisphosphate to fructose-6-phosphate [39]. This conversion relieves the positive allosteric regulation on glycolysis, and thereby, inhibits glycolysis and shunts glycolytic intermediates into the pentose phosphate pathway for NADPH production and glutathione recycling [39]. Consequently, TIGAR expression reduces the rate of glucose breakdown and decreases intracellular ROS. On the other hand, p53 also transcriptionally represses the expression of glucose transporters GLUT1/3/4, and therefore, prevents the uptake of glucose and reduces glycolytic rate [40,41].

More recently, p53 has been implicated in lipid metabolism as well. Indeed, many tumor cells also exhibit high rates of de novo lipid synthesis, and several p53 metabolic targets may possibly counteract such phenomenon via promoting fatty acid oxidation. Jiang et al. identified two repression targets of p53, malic enzyme isoforms 1 and 2 (ME1 and ME2) [42]. ME1 and ME2 catalyze the oxidative decarboxylation of malate in the TCA cycle to pyruvate and NADH/NADPH. Increase in lipid production was observed in p53-deficient cells due to abundance of NADPH, while silencing of malic enzymes

decreases glutamine consumption and glutaminolysis. Interestingly, reduction of ME1/2 expressions can reciprocally activate p53, which led to increased cellular senescence and decreased tumorigenicity of cancer cells, likely through metabolic perturbations that are unfavorable for cancerous growth [42].

In another study, Heffernan-Stroud et al. reported that p53 negatively regulate sphingosine kinase 1 (SK1) via a proteolytic pathway [43]. SK1 is a key enzyme in sphingolipid metabolism that maintains the homeostatic balance of ceramide and sphingosine. Specifically, p53-null mice exhibit increase in SK1 levels that leads to an increase in the pro-growth sphingolipid sphingosine-1-phosphate and a decrease in the anti-growth sphingolipid ceramide [43]. Interestingly, loss of SK1 promotes tumor cell senescence in the thymus of p53-null mice through the elevation of p21 and p16 expressions. Ablation of SK1 reduces tumor formation in p53-null and p53 heterozygous mice, indicating a novel mechanism of p53 tumor suppressing function through sphingolipid regulation [43].

Aside from its function in tumor suppression, p53 also regulate metabolic targets that serve to maintain homeostasis in normal cells and tissues. A recently identified p53 target, Lipin1 is a nuclear transcriptional co-activator that regulates the expression of genes involved in fatty acid oxidation through peroxisome proliferator-activated receptor

alpha (PPAR α) [44]. Under conditions of low glucose, cells upregulate Lipin1 expression through ROS-induced p53 activity and increase fatty acid oxidation to utilize fatty acid as an alternative source of energy [44].

In summary, p53 metabolic targets are involved in increasing mitochondrial respiration, decreasing glycolysis, promoting antioxidant defense and regulating lipid metabolism, all of which could contribute to p53-mediated tumor suppression. Moreover, metabolic functions of p53 further extend outside of tumor suppression to regulate cellular homeostasis in normal physiology, underscoring the multitude of p53 regulation.

1.2.2. Autophagy

Autophagy is a cellular process in which proteins with long half-life and damaged organelles are targeted to lysosome for degradation and recycling. It is a critical pathway for eliminating unwanted cellular components and allowing cells to adapt to nutrient deprivation and hypoxic stress. Recent evidence also linked p53 function to autophagy. While nuclear p53 promotes autophagy through the transcription of pro-autophagic genes, cytoplasmic p53 appears to inhibit autophagy via transcription-independent mechanisms.

DRAM (damage-regulated autophagy modulator) is a lysosomal transmembrane

protein regulated by p53 [45]. Functioning at the crossroad between autophagy and cell death, DRAM induces autophagy downstream of p53, while also contributes to p53-mediated apoptosis. Furthermore, DRAM was found to be down-regulated in certain primary tumors with retention of wild-type p53, suggesting that autophagic regulation by p53 can also contribute to tumor suppression [45].

Several other p53 targets can also promote autophagy via inhibition of the mTOR (mammalian target of rapamycin) axis, a negative regulatory pathway of autophagy. AMP-activated protein kinase (AMPK) is a sensor of cellular energy levels that can promote autophagy by phosphorylating tuberous sclerosis complex (TSC) proteins TSC1 and TSC2, and thereby, inhibits mTOR activity [46]. Interestingly, both the β1 and β2 subunits of AMPK and TSC2 are transcriptional targets of p53, and activation of these targets can induce autophagy [47].

Mechanism of how cytoplasmic p53 can inhibit autophagy is still poorly characterized. However, several evidence demonstrated that either in the absence of nuclear p53 (through enucleation) or in the presence of cytoplasmic-localized mutant p53, inhibition of autophagy persists [48,49]. Furthermore, the degree of autophagic inhibition depended on the ratio of cytoplasmic-to-nuclear p53 levels, with higher cytoplasmic p53 fraction resulting in greater inhibitory effects [49].

1.2.3. Senescence and aging

While cell cycle arrest is transient and reversible, cellular senescence is a permanent aging-related biological process in which cells cease to divide. In normal aging, telomere shortening is the primary initiating event for senescence and aging [50]. On the other hand, persistent oncogenic signaling also triggers a senescence response, also known as oncogene-induced senescence [51]. In either case, genetic instability or DNA damage transpires, and p53 is activated to promote senescence. Studies showed that p21 and plasminogen activator inhibitor-1 (PAI-1) are key effectors of p53-mediated senescence [52-54]. More recently, a novel p53 target E2F7 can also mediate p53-dependent senescence. E2F7 is a transcription factor that represses many genes essential for mitosis, including E2F1, cyclin A, cyclin B, and cdc2/cdk1 [55,56]. The E2F7 transcription factor was shown to be upregulated during oncogene-induced senescence and antagonized oncogenic transformation.

1.3. Post-translational modifications of p53

Unlike many transcription factors whose activities are regulated by synthesis (DNA → mRNA → protein), p53 undergoes numerous post-translational modifications that regulate its stability, activity, and, more recently described, ability to differentially activate

downstream targets. The p53 gene appears to be constitutively expressed under normal physiological conditions, and little is known about whether any mechanism exists to regulate p53 gene transcription. In unstressed cells, however, the p53 protein level is kept at a low steady-state level through ubiquitination of various lysine residues on p53 by E3-ligase Mdm2 and subsequent ubiquitin-mediated proteosomal degradation [57].

Classically, p53 is activated in a three-step process after stress induction. First, p53 is phosphorylated by several kinases, including ATM/ATR/DNA-PK and Chk1/Chk2, which stabilizes p53 by dissociating p53 from its negative regulator Mdm2 [58-60]. Second, stabilized p53 then binds DNA promoters of its downstream target genes. Finally, DNA-bound p53 recruits transcriptional machineries and co-activators to activate transcription of p53 targets. However, recent findings revealed that p53 regulation at the post-translational level are far more complex, which includes a wide array of modifications such as ubiquitination, phosphorylation, acetylation, methylation, sumoylation and neddylation (**Figure 1.3**).

1.3.1. Phosphorylation

Phosphorylation of serine residues on the N-terminal transactivation domain of p53 was the first post-translational modification identified for p53. Phosphorylation at Ser15

Figure 1.3

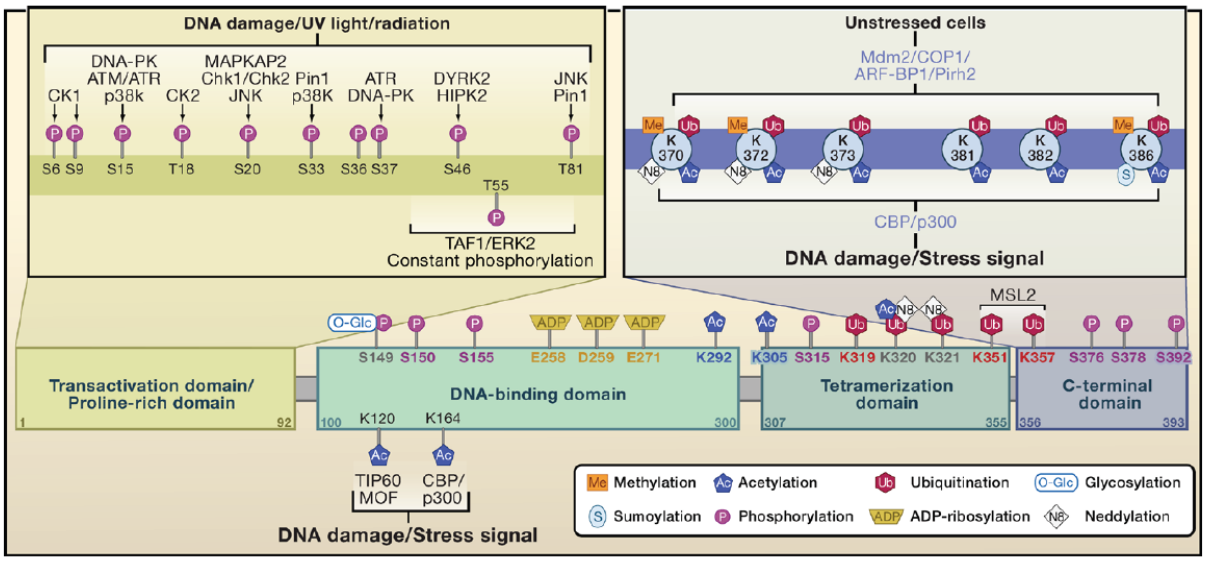

Figure 1.3. Post-translational modifications of p53. The p53 protein is subjected to a vast number of modifications that regulates and fine-tunes its activity and stability, including phosphorylation, ubiquitination, acetylation, methylation, sumoylation and neddylation. (Figure reproduced from Kruse et al, Cell 2009)

(Ser18 in mouse) and Ser20 (Ser23 in mouse) by ATM/ATR/DNA-PK and Chk1/Chk2 kinases was generally thought to be the initial step in p53 activation, which inhibits p53 interaction with Mdm2 [58-60]. Unbound from Mdm2, p53 is thought to be stabilized and readily available to bind DNA and activate transcription.

However, knock-in mouse model that expresses phosphorylation-deficient p53 mutant at Ser18 and/or Ser23 cast doubt on the significance of phosphorylation in p53 activation [61-64]. Using serine-to-alanine point mutations at the N-terminal serine residues of p53, studies showed that single mutations at Ser18 or Ser23 exhibit only modest effects on p53 transcriptional activity, while the degree of p53 stabilization in the mutants is comparable to that of wild-type.

Interestingly, double-mutant mouse model at Ser18/Ser23 revealed greater defect in both p53 level and function, suggesting a synergetic role of these two phosphorylation sites in p53 activation [65]. Nevertheless, the defects observed were not universal in the entire organism, but rather, exhibit in a tissue-specific manner. For example, the transcriptional defects of p53 were mostly limited to thymocytes, while mouse embryonic fibroblasts (MEFs) and other tissues remain unaffected. Furthermore, several studies also demonstrated that p53 can be activated in the absence of N-terminal phosphorylation, suggesting that p53 activation involves a much more complex

regulatory network beyond p53 phosphorylation [66-68].

1.3.2. Ubiquitination

Cellular levels of p53 are tightly regulated to avoid unwanted toxic effects of p53 under non-stressed physiological conditions. Regulation of p53 level is predominantly accomplished through ubiquitin-mediated proteasomal degradation. The p53 protein can be poly-ubiquitinated on lysine residues by several E3-ligases, including Mdm2, COP1, Pirh2, and Arf-BP1 [69-74]. The six C-terminal lysine residues of p53 were first identified to be heavily ubiquitinated by Mdm2 that led to p53 degradation [75]. However, knock-in studies using mouse model with lysine-to-arginine mutations of the C-terminal lysine residues (p53-6KR) showed that the 6KR mutant p53 does not display enhanced stability [76]. Similarly, p53-7KR knock-in mice (seven lysine residues on C-terminal of mouse p53 instead of six lysines on human p53) demonstrate comparable p53 half-life when compared to wild-type mice [77]. These studies established that C-terminal ubiquitination of p53 by Mdm2 is not essential for p53 degradation. Indeed, evidence showed that lysine residues in the DNA-binding domain and the N-terminal may also be ubiquitinated for proteasomal degradation [78]. Additionally, p53 is found to be degraded in the cells of Mdm2-null mice, suggesting that other E3-ligases such as COP1, Pirh2, and Arf-BP1

can contribute to the overall control of p53 levels in cells [79].

Aside from poly-ubiquitination, p53 can also undergo degradation-independent mono-ubiquitination that promotes p53 nuclear export and p53 localization in the cytoplasm [80]. Recent evidence showed that cytoplasmic and mitochondrial p53 play important roles in mediating functions such as apoptosis and autophagy [48,81].

1.3.3. Acetylation

Although acetylation was previously known to only occur on histones, p53 became the first non-histone protein to be functionally regulated by acetylation on lysine residues. The first histone acetyltransferase discovered to acetylate p53 was CBP/p300, and loss of function mutations of CBP/p300 were found in several human cancers [82,83]. CBP/p300 acetylates p53 at the six lysine residues of the C-terminal regulatory domain, which results in the activation of p53 sequence-specific DNA binding [84]. An *in vivo* model of acetylation-deficient p53-6KR knock-in mice showed impaired p53-dependent gene expression in embryonic stem cells and thymocytes after DNA damage, supporting the notion that C-terminal acetylation activates transcriptional activity of p53 [76]. In addition, acetylation of p53 competes with ubiquitination of the same lysine residues by Mdm2 and other E3 ligases, thereby stabilizing p53 and to preventing its

proteosome-mediated degradation [85].

That acetylation of p53 is a crucial mechanism of p53 regulation is further supported by evidence that p53 is deacetylated by deacetylases HDAC1 and Sir2α/Sirt1, and that this reversal of acetylation status significantly represses p53-dependent transcriptional activation. Deacetylation of p53 by the HDAC1 complex modulates apoptosis and growth arrest in cells, and Sirt1-mediated p53 deacetylation represses p53-dependent apoptosis in response to DNA damage and oxidative stress [86-88].

However, while the model that p53 C-terminal acetylation is important in regulating p53 transcriptional activity and the downstream effects of apoptosis and cell growth arrest is well-supported by *in vitro* biochemical studies and certain evidence from an *in vivo* mouse model, the same mouse model also raised concern that the significance of C-terminal acetylation may not be global. Even though, as previously mentioned, embryonic stem cells and thymocytes exhibited dramatic effects from the inability to acetylate C-terminal lysine residues of p53, embryonic fibroblasts derived from the same p53-6KR mice did not show any significant differences from that from wild-type mice [76]. This suggests that C-terminal acetylation may have cell-specific regulatory effects, rather than a general impact on apoptosis or cell cycle control.

In addition to CBP/p300, p300/CBP-associating factor (PCAF) is another

acetyltransferase that acetylates p53 at lysine K320 [89,90]. Acetylation of K320 was found to be increased after DNA damage and enhanced DNA-binding of p53 to target promoters. However, analysis of the K317R (analogous to K320 in human) p53 mutant mice revealed unexpected results. Instead of disrupting the activation of p53, the K317R mutant p53 appeared to increase the expression of pro-apoptotic genes in mouse thymocytes and intestinal epithelial cells, indicating that the function of the K320 acetylation may be more complex than previously hypothesized [91].

Although the lysine mutant knock-in mouse model casts doubt on the importance of C-terminal acetylations, recent studies have shown that p53 acetylation is not limited to the C-terminal domain. Two independent studies showed that the lysine residue K120 of the DNA-binding domain is acetylated by two different acetyltransferases – Tip60 and hMOF [92,93]. Tip60 and hMOF belong to the MYST family of acetyltransferases that share no homology with the CBP/p300 acetyltransferases. The K120 lysine residue is conserved in all species with functional p53 gene, implying that acetylation at this site may play an evolutionarily conserved role. More importantly, Tip60 was identified as a p53 activator from a large-scale RNA interference screen [94].

Acetylation of the K120 site is essential for the p53-mediated apoptotic response to DNA damage. Expression of the proapoptotic target genes Puma and Bax requires the

K120 acetylation of p53, while expression of p21 and Mdm2 do not. When the K120 lysine residue is mutated to arginine, p53-mediated expression of Puma and Bax genes are impaired, while the expression of Mdm2 is not affected and that of p21 gene is only slightly decreased. Furthermore, chromatin-immunoprecipitation (ChIP) using wild-type p53 and acetylation-deficient mutant p53-K120R showed that both wild-type and mutant p53 are recruited to the promoters of Puma, Mdm2 and p21 equally, implying that the K120 acetylation does not affect DNA binding ability of p53 [93].

Another recently identified acetylation site in the DNA-binding domain is K164, acetylated by CBP/p300 [95]. Simultaneous loss of acetylation at K164, K120, and the six C-terminal lysine residues (p53-8KR) completely abolishes the ability of p53 to induce p21 expression and cell cycle arrest. However, loss of acetylation individually at the aforementioned lysine residues still allows p53 to activate p21, suggesting that these acetylation sites may compensate one another through redundancy. Notably, the p53-8KR mutant retains DNA-binding capabilities and is able to transactivate the Mdm2 negative-feedback loop.

1.3.4. Methylation, Sumoylation, and Neddylation

Lysine residues are very versatile in their ability to be post-translationally modified.

Besides ubiquitination and acetylation, as previously described, lysine residues on p53 can also undergo methylation, sumoylation and neddylation.

Three different methyltransferases have been reported to methylate p53 on the C-terminal lysine residues. Monomethylation by Set7/9 at lysine K372 promotes p53 activity and enhances transactivation of p21 [96]. On the other hand, monomethylation at K370 and K382 by Smyd2 and Set8/PR-Set7, respectively, represses p53 transcriptional activity [97,98]. Interestingly, K370 of p53 can also be dimethylated, although by an unknown methyltransferase, and the dimethylated lysine residue act as an anchor for binding to co-activator 53BP1 (p53-binding protein 1). Demethylase LSD1 preferentially removes the dimethylation on K370, and thereby, inhibits the interaction between p53 and 53BP1 and represses p53 activity [99].

Sumoylation is the addition of ubiquitin-like SUMO substrate on lysine residue. p53 has been shown to be sumoylated at K386. While one study demonstrated that sumoylation activates p53, another study reported that this modification promotes cytoplasmic localization of p53 [100,101].

Neddylation, on the other hand, appears to inhibit p53 transcriptional activity. Neddylation, the conjugation of ubiquitin-like NEDD8 molecule, can be mediated by Mdm2 at lysine residues K370, K372, and K373 [102]. In another study, neddylation was

observed at K320 and K321 by FBXO11 [103].

1.4. Mechanism of p53-mediated tumor suppression

Although the molecular mechanism of how p53 achieves tumor suppression is not entirely understood, it has long been accepted that tumor proliferation can be inhibited by p53-mediated apoptosis, cell growth arrest and senescence. Indeed, pausing cell cycle progression to repair DNA damage or ultimately terminating a cell harboring irreversible genotoxic damage do prevent the accumulation of oncogenic mutations in an organism. However, recent evidence suggests that tumor suppression by p53 can be achieved in the absence of p53-mediated apoptosis, cell growth arrest and senescence.

1.4.1. Apoptosis and growth arrest are not essential in tumor suppression

The earliest evidence came from the analysis of the p21/WAF1 knock-out mouse model in which no spontaneous tumorigenesis was observed [104,105]. This may not come as a surprise, as apoptotic functions of p53 may very well compensate for the lack of growth arrest. Later studies also failed to observe any increased tendency in tumor formation in apoptosis-deficient Puma and Noxa double knock-out mice, possibly due to intact growth arrest function [23,106]. Surprisingly, however, the $p53^{3KR/3KR}$ knock-in

mice (loss of acetylation at lysine residues K117 and K161/162 via replacement with arginine), which exhibit defects in both p53-dependent apoptotic and cell cycle arrest response, do not present with significantly earlier onset of tumorigenesis compared with wild-type mice [107]. While p53-null mice succumb to early onset of thymic lymphomas before the age of 6-months, most $p53^{3KR/3KR}$ mice remain healthy up to 16-months of age. These studies suggest that properties of p53 other than apoptotic and growth arrest functions may contribute to p53-mediated tumor suppression. Interestingly, $p53^{3KR/3KR}$ mice retain the ability to regulate certain metabolic genes, including TIGAR and GLS2. Recent evidence has implicated p53 metabolic targets, such as TIGAR and GLS2, in tumor suppression through their ability to modulate the metabolic state of cancer cells [37,39].

Similarly, mouse model containing mutations in an N-terminal transactivation domain at residues 25 and 26 ($p53^{25,26/25,26}$) fail to upregulate downstream apoptotic and growth arrest targets, but retain the ability to prevent tumorigenesis [108]. Finally, triple knock-out mice deficient in p21, Puma and Noxa expressions do not develop spontaneous tumors [109]. Collectively, these pieces of evidence underscore the potential significance of p53 non-canonical functions in contributing to p53-mediated tumor suppression.

1.4.2. Role of p53 metabolic regulation in tumor suppression

As described in previous sections, p53 metabolic targets have functions that counteract the metabolic alterations commonly seen in cancer cells. Indeed, as evident in the $p53^{3KR/3KR}$ mice, metabolic regulation by p53 could potentially play critical role in suppressing tumor formation [107]. However, with yet another twist in this convoluted story, p53 metabolic targets may not always function as tumor suppressors.

Evidence supporting p53 metabolic targets as tumor suppressors showed that loss of GLS2 expression correlates with neoplastic transformation in human hepatocellular carcinomas [36,37], while low SCO2 expression is a factor of poor prognosis in patients with breast cancer [110]. The rationale behind this is two-fold: 1) cancer cells display a heightened state of aerobic glycolysis that confers several growth advantages over normal cells, and p53 targets such as SCO2, TIGAR and GLS2 may contribute to p53-mediated tumor suppression by preventing such metabolic transformation; and 2) upregulation of antioxidant production by p53 metabolic targets prevents the increase in oxidative stress associated with malignant transformation.

On the other hand, while aerobic glycolysis promotes rapid growth in tumors, it is not without pitfalls. The inefficiency of glycolysis for energy generation could become perilous for cancer cells that have outgrown the resource capacity of their tumor

environments. Similarly, although cancer cells generally have increased levels of ROS, which perpetuates cell proliferation and are involved in tumor initiation and progression, excessive accumulation of ROS is also toxic to cancer cells. Evidence showed that cancer cells require robust antioxidant capacity to offset the intrinsic oxidative stress for survival [111-114]. Given that p53 metabolic targets have the capability of modulating energy homeostasis and decreasing intracellular ROS levels, they could potentially support cancerous growth under certain circumstances. Nevertheless, precisely how these targets affect tumorigenicity or survival of cancer cells remains elusive.

Recent studies have attempted to further our understanding of the function of p53 metabolic targets in the context of tumor biology, but the findings only broadened the complexity of tumor metabolism. Current evidence demonstrates that certain cancers gain survival advantages by retaining wild-type p53 and the subsequent activation of p53 metabolic targets. Wanka et al. showed that glioma and colon cancer cells that express wild-type p53 are less sensitive towards hypoxia-induced cell death [115]. These cancer cells were able to maintain mitochondrial respiration under hypoxic conditions through the activation of SCO2, while cells depleted of p53 or SCO2 undergo necrotic cell death in the presence of low oxygen [115]. TIGAR is also reported to have tumor-protective functions as well. Cheung et al. reported that TIGAR plays a role in enhancing intestinal

adenoma proliferation as well as regeneration of intestinal epithelium [116]. Through several rescue-experiments, the authors concluded that Tigar expression prevents ROS accumulation through increasing GSH:GSSG ratio and increases nucleotide synthesis that allow intestinal tumor cell to grow more efficiently. Indeed, tumor burden is decreased in TIGAR knock-out mice compared to TIGAR wild-type mice using an intestinal adenoma mouse model [116]. Similarly, another study by Wanka et al. revealed that Tigar expression protects glioma cells from glucose starvation and hypoxia-induced cell death, which coincides with TIGAR been overexpressed in glioblastomas [117]. Together, these studies provide evidence that p53 may protect tumor cells from their harsh microenvironment or oxidative stress through the regulation of metabolic targets, which are also suggested by previous studies [118,119].

These findings present a rather puzzling question – does p53 suppress tumor formation or does it enhance tumor cell survival? The answer, it seems, is that p53 may act as a double-edged sword (**Figure 1.4**). While metabolic functions of p53 do indeed contribute to the overall tumor suppressing activity of p53, they also serve to protect cells, normal or tumor alike, from metabolic stress. By lowering ROS levels and maintaining energy homeostasis, p53 metabolic targets may avert crises that would otherwise result in cell death. For example, while normal healthy cells must maintain oxidative balance for

Figure 1.4

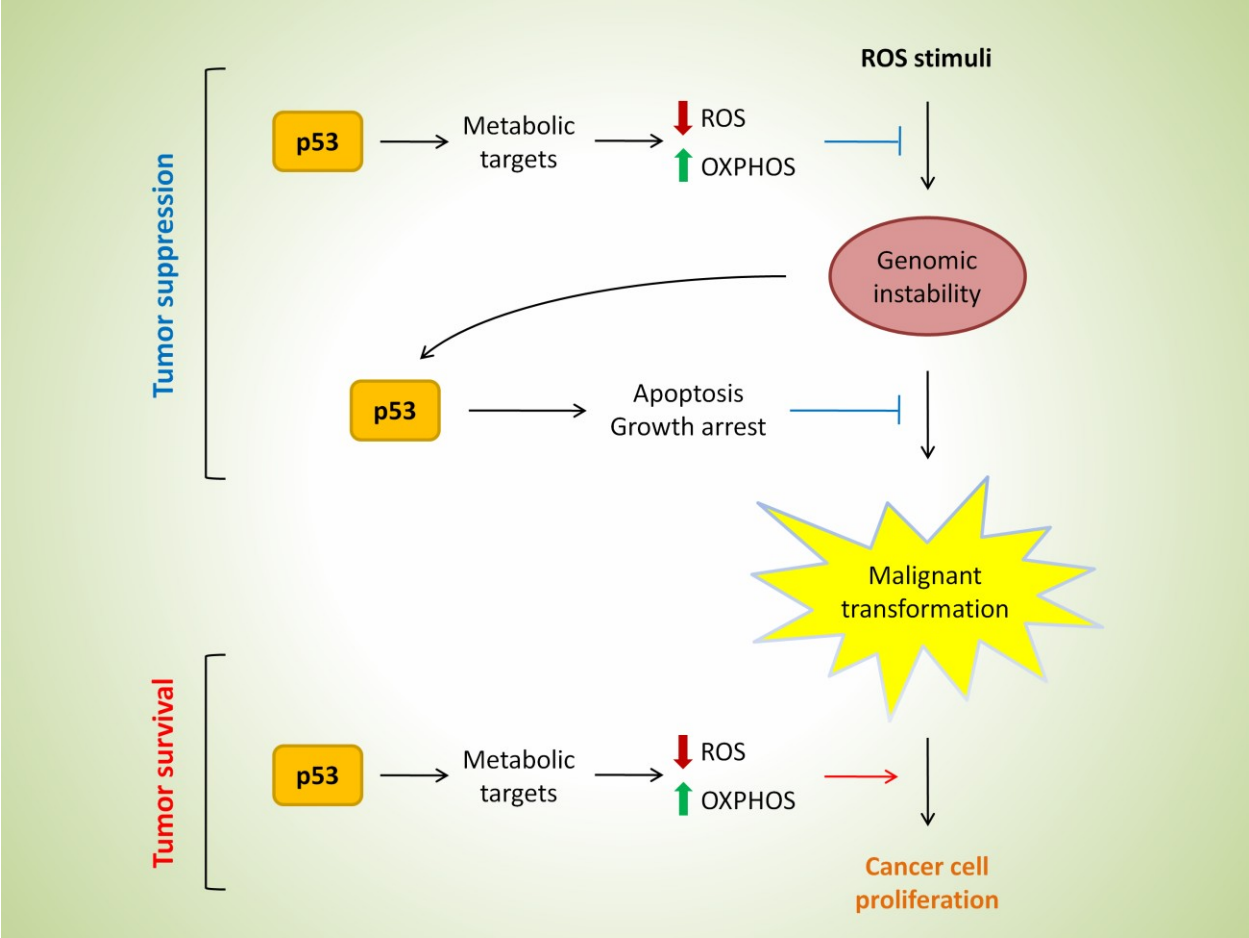

Figure 1.4. Opposing functions of p53 metabolic regulation. Metabolic regulation by p53 in tumorigenesis is a double-edged sword: maintaining oxidative balance and energy homeostasis could prevent cancer development in the pre-cancerous stage, but favors tumor survival after malignant transformation.

survival, cancer cells have an even lower threshold for oxidative insults [34]. In order for tumor cells to thrive, they must upregulate pathways for antioxidant production, many of which are controlled by p53 targets. Moreover, tumor cells often suffer from hypoxic and nutrient stress due to rapidly growing cells competing for finite amount of resources, especially for cells residing in the center of the tumor bulk where blood supply is limited. Under such conditions, tumor cells will benefit from a shift from aerobic glycolysis to the higher efficient oxidative phosphorylation for energy production, which is also regulated by p53 activity. If indeed p53 function manifests such duality, then when in the evolution of normal to tumor cells does p53 seizes to exist as a tumor suppressor and don the role of enhancing tumorigenicity? Further studies are warranted in order to dissect the intricate balance of cell death and survival in p53 function on tumorigenesis.

1.5. Differential regulation of p53 targets

With the ever-expanding number of p53 targets involved in a diverse array of cellular processes, it is crucial for p53 to selectively activate its downstream targets to achieve the desired outcome depending on the cellular context. Currently, the mechanism underlying the preferential expression of p53 targets remains largely unknown. Previous evidence, however, points towards the possibility that the acetylation of specific lysine

residues of p53 can differentially regulate the expression of pro-apoptotic and growth arrest genes. Acetylation of p53 at the K120 lysine residue by acetyltransferases Tip60/hMOF is crucial for p53-mediated apoptosis but not for cell cycle arrest [92,93]. On the other hand, p53 acetylation at K164 is acetylated by CBP/p300, and loss of acetylation at both K120 and K164 abolishes the ability of p53 to induce both apoptosis and growth arrest [95]. This notion is also supported by the 3KR mouse model. While p53-mediated apoptotic and growth arrest gene expressions are abrogated in the 3KR mice, expression of Mdm2 and many of the metabolic targets are still retained [107]. These findings suggest that additional post-translational modifications of p53 may be required for the preferential expression of p53 metabolic and non-canonical genes.

Given our current knowledge, we hypothesize that the regulation of downstream targets by p53 may be hierarchical in nature (**Figure 1.5**). Depending on the severity and the type of stress in question, specific sites and number of p53 modifications may vary in which to dictate the appropriate p53 response. Under no or low stress conditions, minimal post-translational modifications of p53 may be sufficient to activate targets such as Mdm2 and various metabolic genes. In the absence of stress, expression of Mdm2 is required to negatively regulate p53 activity in order to avoid p53-induced cell death. Meanwhile, under low stress conditions such as starvation and hypoxia, activation of

Figure 1.5

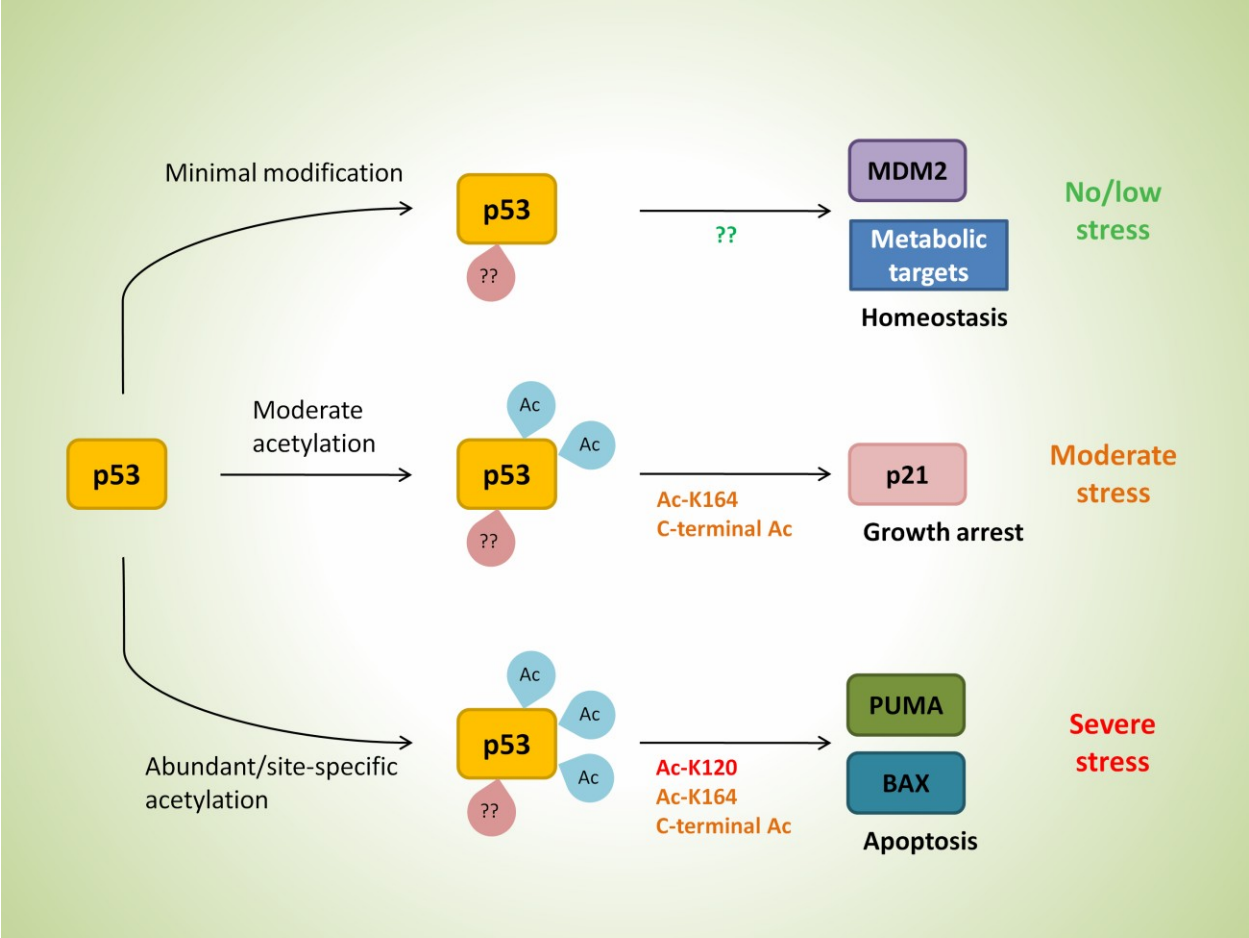

Figure 1.5. A hypothetical model of differential regulation by p53. p53 is subjected to a hierarchical sequence of post-translational modifications as stress level increases, which induces various functional outcome depending on the combination of modifications (see text for complete description).

metabolic targets could enhance cell adaptation and survival. Upon facing moderate stress, however, p53 acetylation increases at K164 and C-terminal lysine residues that lead to growth arrest via p21 induction, which allow cells to recover and repair cellular damage. Ultimately, if the damage from stress becomes irreparable, acetylation at the K120 site would trigger an apoptotic response to circumvent the perpetuation of possible genetic lesions.

The rationale underlying our hypothesis is that p53 metabolic targets, unlike its canonical counterparts involved in apoptosis and growth arrest, are relatively harmless in nature and serve to improve cellular homeostasis, and therefore, are subjected to a hierarchical regulation. This would allow the selective expression of metabolic genes and Mdm2 in the presence of basal p53 expression under normal growth without activating other p53 functions. It is also possible that p53 targets functioning in oxidative balance and autophagy may be regulated in a similar fashion. Since mounting evidence suggests that non-canonical functions of p53 are critical in tumor suppression and/or cell survival and homeostasis, elucidating the minimal modifications for activating p53 non-canonical targets could be the key to understanding the mechanism of how p53 participate in cellular processes such as tumorigenesis and adaptation.

2. Research Description

2.1. Chapter 1: p53-dependent regulation of metabolic function through transcriptional activation of pantothenate kinase-1 gene

2.1.1. Background and Rationale:

The p53 protein is a tumor suppressor whose primary function is to maintain the integrity of the genome through cell fate control [1] (see Section 1.1). Classically, p53 is activated under genotoxic stress and acts as a transcription factor to regulate the expression of downstream apoptotic or growth-arrest targets [13,14]. However, recent works have shown that functions of p53 go far beyond cell death and growth arrest, and now also include metabolism, autophagy, senescence and aging [24,25] (see Section 1.2).

Proper metabolic function is crucial to the viability of normal cells. Interestingly, metabolism also plays a key role in the adaptability of neoplastic cells, the process of tumorigenesis, and genome stability, and p53 has been recently linked to these aspects of metabolic functions [120]. p53 has been shown to regulate the balance between glycolysis and mitochondrial respiration through transcriptional control of several metabolic genes. SCO2 gene, a transcription target of p53, codes for a protein that assembles and regulates the cytochrome C oxidase complex of the electron transport chain. Synthesis of the Sco2 protein increases cellular mitochondrial respiration as a

mean of energy production, while decreasing the need for glycolysis. Interestingly, loss of p53 in cancer cells correlates with decreased SCO2 expression, which leads to a shift of energy production towards glycolysis, a phenomenon known as the Warburg's Effect [35]. p53 also represses the expression of glucose transporters GLUT1/3/4, thereby decreasing cellular uptake of glucose and subsequent glycolysis [40]. In addition, p53 negatively regulates the transcription of the pyruvate dehydrogenase kinase PDK2, which phosphorylates and inhibits pyruvate dehydrogenase (PDH). By relieving the inhibitory effect on PDH, p53 facilitates the conversion of pyruvate into acetyl-CoA by PDH as substrate for mitochondrial respiration [121].

Furthermore, p53 has been implicated in metabolic pathways that regulate the synthesis of cellular antioxidant glutathione, which protects cells against reactive oxygen species (ROS). TIGAR, a p53 target, inhibits glycolysis by decreasing the level of fructose-2,6,-bisphosphate and shunts glucose into the pentose phosphate pathway for NADPH production that leads to glutathione recycling [39]. To a similar effect, p53 can upregulate the expression of glutaminase GLS2, which converts glutamine to glutamate for glutathione synthesis [36,37].

Aside from genotoxic stress, cells may also activate p53 under metabolic stress. Studies have shown that p53 is induced under energy and nutrient deprivation by

energy-sensing AMP-activated protein kinase (AMPK), and may lead to subsequent activation of growth arrest pathways [122,123]. In addition, p53 also induces autophagy in the face of prolonged starvation to generate sufficient energy for survival through regulation on LC3 [124]. Together, these evidence suggest that p53, besides being a tumor suppressor, may also play a prosurvival role of maintaining metabolic homeostasis under energy crisis.

In light of these recent evidence that point towards the significance of p53 in metabolic regulation, we performed a chromatin-immunoprecipitation-on-chip (ChIP-on-chip) analysis using p53 as bait and identified pantothenate kinase-1 (*PANK1*) as a potential metabolic target of p53. PanK1 is a member of a family of proteins (PanK family) that are involved in the biosynthesis of coenzyme A (CoA). CoA is an essential and ubiquitous cofactor that is obligatory in over 100 different intermediary metabolic reactions, including those involved in fatty acid metabolism, tricarboxylic acid cycle, and cholesterol and bile acid synthesis. The PanK family of proteins catalyze the first and rate-limiting step of CoA synthesis (phosphorylation of precursor pantothenate), and thus control the cellular content of CoA [125].

There are two PanK isoforms encoded by the *PANK1* gene, PanK1α and PanK1β, and two other isoforms (PanK2 and PanK3) encoded by two other distinct genes. PanK1

is most highly expressed in the liver, and corresponds to the liver possessing the highest concentration of CoA among tissues [126]. This is consistent with the fact that a plethora of tissue-specific metabolic functions of the liver require CoA, which includes β-oxidation, ketogenesis, gluconeogenesis, and sterol synthesis. CoA levels in tissues, especially in the liver, change under metabolic stress in order to utilize alternative fuel sources to meet energy demands. In cases such as starvation and diabetes, increase in the liver intracellular CoA concentration is required to promote sufficient conversion of stored fatty acids and amino acids into ketone bodies and glucose to supply the rest of the body [127].

Here, we report that PanK1 is a transcriptional target of p53, but does not contribute to p53-dependent apoptosis or growth arrest. Instead, we observed that p53 is required to maintain PanK1 expression *in vitro* after glucose starvation and in the mouse liver during fasting. In the absence of p53, PanK1-dependent CoA synthesis decreases, leading to impediment of catabolic and anabolic processes in the liver.

2.1.2. Materials and Methods:

Cell Culture, Transfection, siRNA-Mediated Knockdown and Antibody. H1299 and U2OS cells were maintained in DMEM and HCT116 cells in McCoy's 5A medium. All

media were supplemented with 10% fetal bovine serum. Transfections with plasmid DNA and siRNA were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Ablation of PanK1 was performed by transfection of HCT116 cells with siRNA duplex targeting human PanK1 mRNA (5' – GUGGAACGCUGGUUAAAUU – 3'). The rabbit polyclonal antibody specific for PanK1 protein was produced in collaboration with Bethyl Laboratories, Inc.

Chromatin Immunoprecipitation (ChIP). Adherent cells were incubated in culture media containing 1% formaldehyde with gentle shaking for 10 min at room temperature, and crosslinking was stopped by addition of 2.5 M glycine to a final concentration of 0.125 M glycine. After two washes with cold PBS, cells were harvested in ice cold lysis buffer (10 mM Tris-Cl [pH 8.0], 85 mM KCl, 0.5% NP-40, 5 mM EDTA, 0.25% Triton-X, and fresh proteinase inhibitor cocktail) and incubated on ice for 10 min. Nuclei were collected, suspended in cold RIPA buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.1% DOC, 1% Triton X-100, 5 mM EDTA, and fresh proteinase inhibitor cocktail), and sonicated to shear the genomic DNA to an average of 300 bp. Cleared extracts were blocked with protein A beads (Millipore), and p53 proteins were immunoprecipitated by FL-393 p53 antibody (Santa Cruz) followed by binding of antibodies to protein A beads.

After eight washes by RIPA buffer with gentle rotation for 5 min each time, the proteins were eluted from the beads by 0.5 mL elution buffer (30 mM Tris-Cl [pH 8.0], 15 mM EDTA, 200 mM NaCl and 1% SDS). The DNA samples were recovered by phenol extraction and ethanol precipitation after reversal of crosslinking. The purified DNA were then analyzed either by PCR within linear amplification range followed by agarose gel electrophoresis or by quantitative real-time PCR using Applied Biosystems 7500 Fast System. The following primers were used for PanK1 ChIP: forward primer 5' – CGATGCCCATCTGGTTTTCACATCG – 3' and reverse primer 5' – GCCACCGACGAGTTTCAACA – 3'.

Luciferase Activity Assay. Promoter-containing fragments were amplified from human genomic DNA of H1299 cells and cloned into the pBVLuc luciferase reporter vector containing a minimal promoter (He et al., 1998). To test the potential p53 binding sites, fragment for pLucA containing the p53 BS was amplified using forward primer 5' – GATGCCGCCTTCCCTTCTTA – 3' and reverse primer 5' – GAGGAAGCCGCGTTTGAAGT – 3', while fragment for pLucB without the p53 BS was amplified using the same forward primer and reverse primer 5' – CATCGCCATACAAAGCCCAA – 3'. Mutations of the various p53 binding sites were

generated using site-directed mutagenesis with the following oligos: 5' – CCCATCTCCTCTCTGTAATCCCAGAGAACTTGAT – 3' and 5' – ATCAAGTTCTCTGGGATTACAGAGAGGAGATGGG – 3' for pLucA'1, 5' – CCAGAGAATTTTATTCCCTATATTGAAACTCGTCGGTGGC – 3' and 5' – GCCACCGACGAGTTTCAATATAGGGAATAAAATTCTCTGG – 3' for pLucA'23, and 5' – GGCGGTGGCGGGTAGTACTGGGAATTCTCAGTGGCGGG – 3' and 5' – CCCGCCACTGAGAATTCCCAGTACTACCCGCCACCGCC – 3' for pLucA'45. Transfection of H1299 cells were performed in 24-well plate using 0.2 μg luciferase reporter constructs, 0.05 μg p^{RL}-tk Renilla construct, and various amount of p53-expressing vector. Luciferase activities were measured 24 hrs post-transfection using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized with Renilla luciferase activities to obtain the relative luciferase activity.

Gel Mobility Shift Assay (EMSA). Purified Flag-p53 proteins were obtained from transfected 293 cells. The 171-bp DNA probe containing the p53 binding sites were PCR-amplified from *PANK1* promoter using primers 5' – CTGTCTCTCCCAGACCCAT – 3' and 5' – AGTCTGGGAGGCGAGGAAG – 3', labeled by T4 kinase (NEB, M0201S) and purified using the Bio-Spin 30 columns (Bio-Rad). The protein-DNA binding

reactions (20 µl) contained 20 mM HEPES (pH 7.6), 80 mM NaCl, 0.1 mM EDTA, 12.5% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.7 mM DTT, 200 ng/µL BSA, 20 ng/µL sheared sperm DNA, 10–20 fmol DNA probe, and 200 ng Flag-p53. In supershift assays, 200 ng pAb421 (Millipore) antibody was added to the reaction. Wild-type and mutant competition probes were PCR-amplified from pLucA and pLucA'2345, respectively, using the above primers.

RNA Extraction and RT-qPCR. Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse Transcriptase kit (NEB). PCR analysis was carried out using Applied Biosystems 7500 Fast System with the following primers: 5' – CGCTGCGAAATACACCAATTTAACCAGCG – 3' and 5' – AAGAACAGGCCGCCATTCC – 3' for human PanK1α, and 5' – ACAGGACGCTGTGGGATGTAAA – 3' and 5' – CGCTGCGAAATACACCAATTTAACCAGCG – 3' for human PanK1β.

PanK Activity Assay. Enzyme preparation and assays were performed as described previously (Vallari et al., 1987). The pantothenate kinase specific activities in cell lysates

were calculated as a function of protein concentration measured by Bradford method. Assays contained d-[1-^{14}C]-pantothenate (45.5 μM; specific activity 55 mCi/mmol), ATP (2.5 mM, pH 7.0), $MgCl_2$ (2.5 mM), Tris–HCl (0.1 M, pH 7.5), and increasing amounts of cell extract in a total volume of 40 μL. The mixture was incubated for 10 min at 37°C and the reaction was stopped by depositing a 30 μL aliquot onto a Whatmann DE81 ion-exchange filter disk that was washed with 1% acetic acid in 95% ethanol (25 ml/disk) three times to remove non-reacted pantothenate. 4' – Phosphopantothenate was quantified by counting the dried disk in 3 mL scintillation solution.

Free CoA and Long-Chain Acyl-CoA Measurements. Extraction of free CoA and long chain acyl-CoA were performed as described previously. Briefly, 100 mg of mouse tissue was homogenized in 0.4 mL chilled 6% perchloric acid containing 28 mM DTT. After centrifugation (1500 × g, 4°C, 10 min), the precipitate contains the acid-insoluble long-chain acyl-CoAs, while the supernatant contains the acid-soluble free CoA. For free CoA extraction from the supernatant, 0.1 volume of 1 M Tris and 0.3 volume of 2 M KOH were added before the pH was adjusted to 6.5~8.5 with 0.6 N HCl. The mixture was centrifuged (1500 × g, 4°C, 10 min) and the supernatant was saved for CoA determination. For long-chain acyl-CoA extraction, the precipitated pellet was washed

with 0.6% perchloric acid containing 5 mM DTT and then washed with 5 mM DTT. The washed pellet was resuspended in 300 µL of 5 mM DTT and the pH was adjusted to 11∼12 with 1 M KOH. The mixture was incubated at room temperature for 1 hr, neutralized with 0.6 N HCl to pH 6.5∼8.5, and centrifuged (1500 × g, 4°C, 10 min). The resultant supernatant was extracted with an equal volume of hexane three times before being used in the CoA determination assay. The reaction mixture for the CoA determination assay contained 200 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 0.1% Triton X-100, 2 mM EDTA, 20 mM NaF, 2.5 mM ATP, 10 µM [1-^{14}C]lauric acid, 100 ng of *E. coli* acyl-CoA synthetase (FadD), and tissue extract (5–20 µL) in a total volume of 100 µL. The reaction was initiated by the addition of FadD, and the reaction mixture was incubated at 35°C for 30 min, followed by the addition of 325 µL of methanol:chloroform:n-heptane (1.41:1.25:1, v/v/v) and 25 µL of 0.4 M acetic acid. After the mixture was mixed vigorously and centrifuged, [1-^{14}C]lauroyl-CoA in the upper phase was quantified by counting in 3 mL scintillation solution.

Oil Red O Staining. ORO staining was performed by Columbia University HICCC Histology Services. The mouse liver frozen sections were cut at 7 µm thickness and fixed in 10% formalin before staining with Oil Red O solution and Hematoxylin.

Pyruvate Challenge Test and Blood Glucose Measurements. For the pyruvate challenge test, mice were starved for 16 hrs and were then intraperitoneally injected with pyruvate dissolved in saline (2 mg/g of body mass). Blood glucose was measured using OneTouch Ultra Blue Test Strips (LifeScan Inc.).

2.1.3. Results and Discussion:

PANK1 gene is a p53 transcriptional target

PANK1 promoter was identified in a ChIP-on-chip assay using p53-transfected H1299 cells. The potential p53 binding site (BS) on *PANK1* promoter was approximated to the 5'-end of exon 1 of *PANK1α* isoform (**Figure 2.1**). To verify the binding of p53 to the *PANK1* promoter, we carried out chromatin-immunoprecipitation (ChIP) in H1299 cells transfected with p53 expression vector, followed by quantitative real-time PCR (qPCR) amplification of the pulled-down DNA fragments. The relative p53 enrichment at the potential *PANK1* p53 BS was similar to those at promoters of well-established p53 metabolic targets (TIGAR, SCO2, and GLS2), while no enrichment was detected at the region 2kb upstream of the potential *PANK1* p53 BS, proving that p53 indeed binds to the *PANK1* promoter (**Figure 2.2**).

Since p53 is a transcription factor, we tested whether or not p53 is able to activate

Figure 2.1

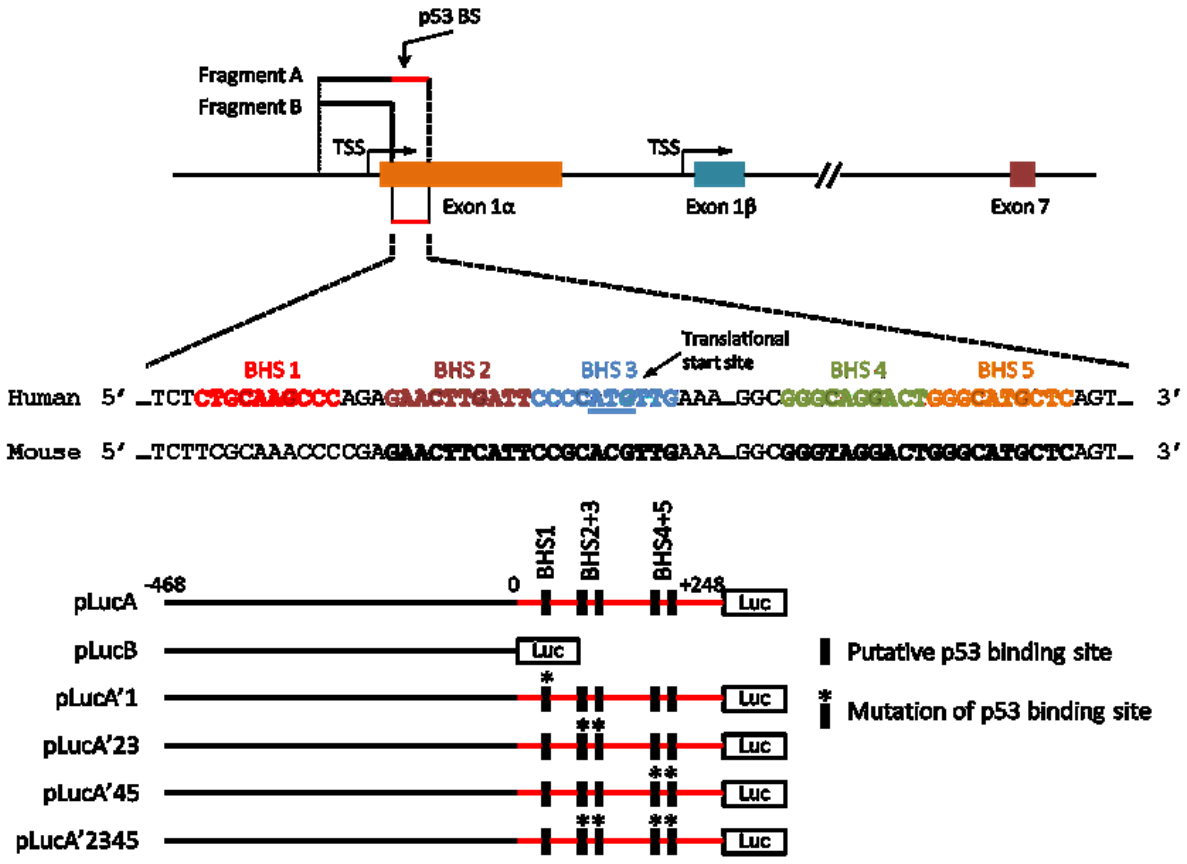

Figure 2.1. Schematic representation of the human *PANK1* gene and its promoter. The potential p53 binding sites (p53 BS) are located in the 5' region of *PANK1* exon 1α. TSS represents the transcriptional start site. Luciferase construct pLucA containing the p53 BS and construct pLucB lacking the p53 BS are derived from the potential *PANK1* promoter region for luciferase reporter gene assays in C and D. In addition, luciferase constructs containing mutations of the potential p53 consensus sequences are denoted as pLucA'1, pLucA'23, pLucA'45, and pLucA'2345.

Figure 2.2

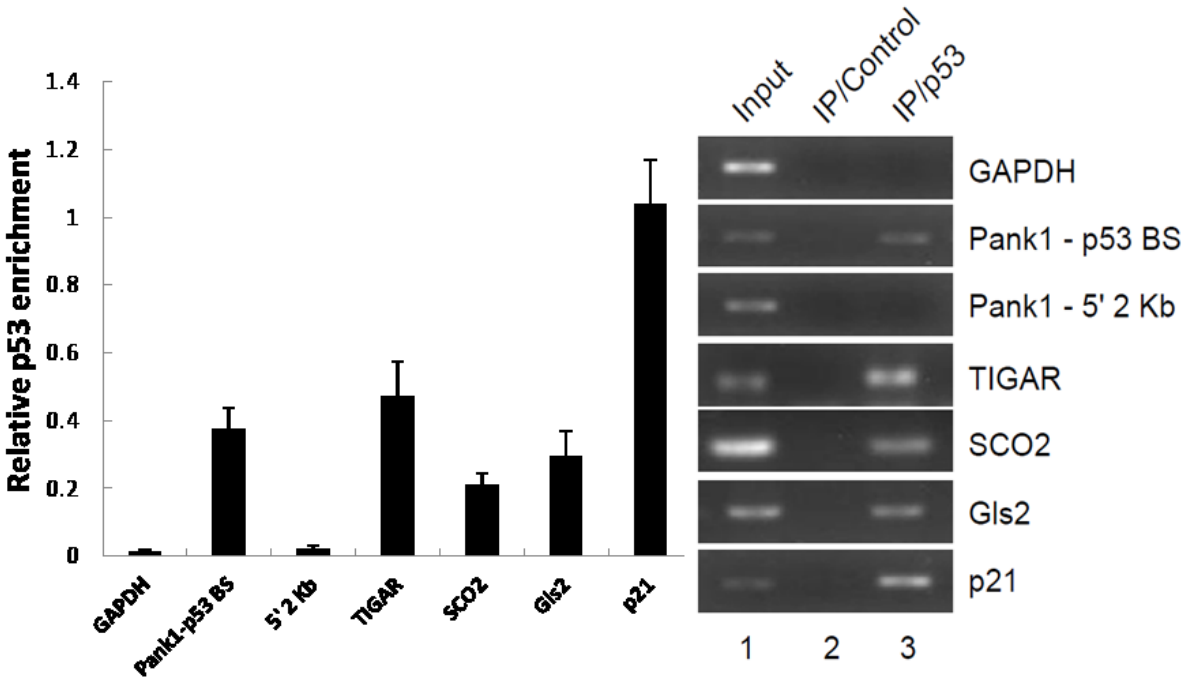

Figure 2.2. Validation of p53 binding on *PANK1* promoter. The left panel shows ChIP-qPCR analysis of p53 enrichment at the promoter regions of *PANK1*, *TIGAR*, *SCO2*, *GLS2*, and p21 in H1299 cells expressing p53. *GAPDH* and a region located 2-kb upstream of the *PANK1* p53 BS were used as negative controls. The right panel shows semi-quantitative analysis of p53 binding to the corresponding regions in the left panel.

transcription through the *PANK1* promoter using luciferase assay. Luciferase constructs pLucA containing Fragment A of *PANK1* promoter (as depicted in Figure 2.1) with the potential p53 BS and pLucB containing Fragment B without the p53 BS were generated. Co-transfection of pLucA with WT p53 expression vector into p53-null H1299 cells increased luciferase activity in a p53 dose-dependent manner, while co-transfection with binding-deficient mutant p53-R175H failed to do so (**Figure 2.3A**). Interestingly, co-transfection of pLucB with p53 expression vector did not induce reporter activity, confirming that the potential p53 binding site is indeed at the predicted region.

Five potential p53 binding half-sites (BHS) were found at the 5' region of *PANK1* exon1α that overlaps with the translational start site (**Figure 2.1**). Mutation of each BHS was generated by mutating the most conserved C/G to A/T (RRR**C**WW**G**YYY → RRR**A**WW**T**YYY) in pLucA. Mutation of BHS 1 did not reduce transcriptional activity in the presence of p53, while mutations of BHS 2+3 and BHS 4+5 significantly reduced p53-dependent transcriptional activity (**Figure 2.3B**). Furthermore, mutating all four binding half-sites completely abrogated reporter transcriptional activity by p53 (**Figure 2.3B**).

We next performed gel mobility shift assay to determine whether p53 binds to the four BHS. Using immunoprecipitated Flag-tagged p53, we observed a shift using a

Figure 2.3

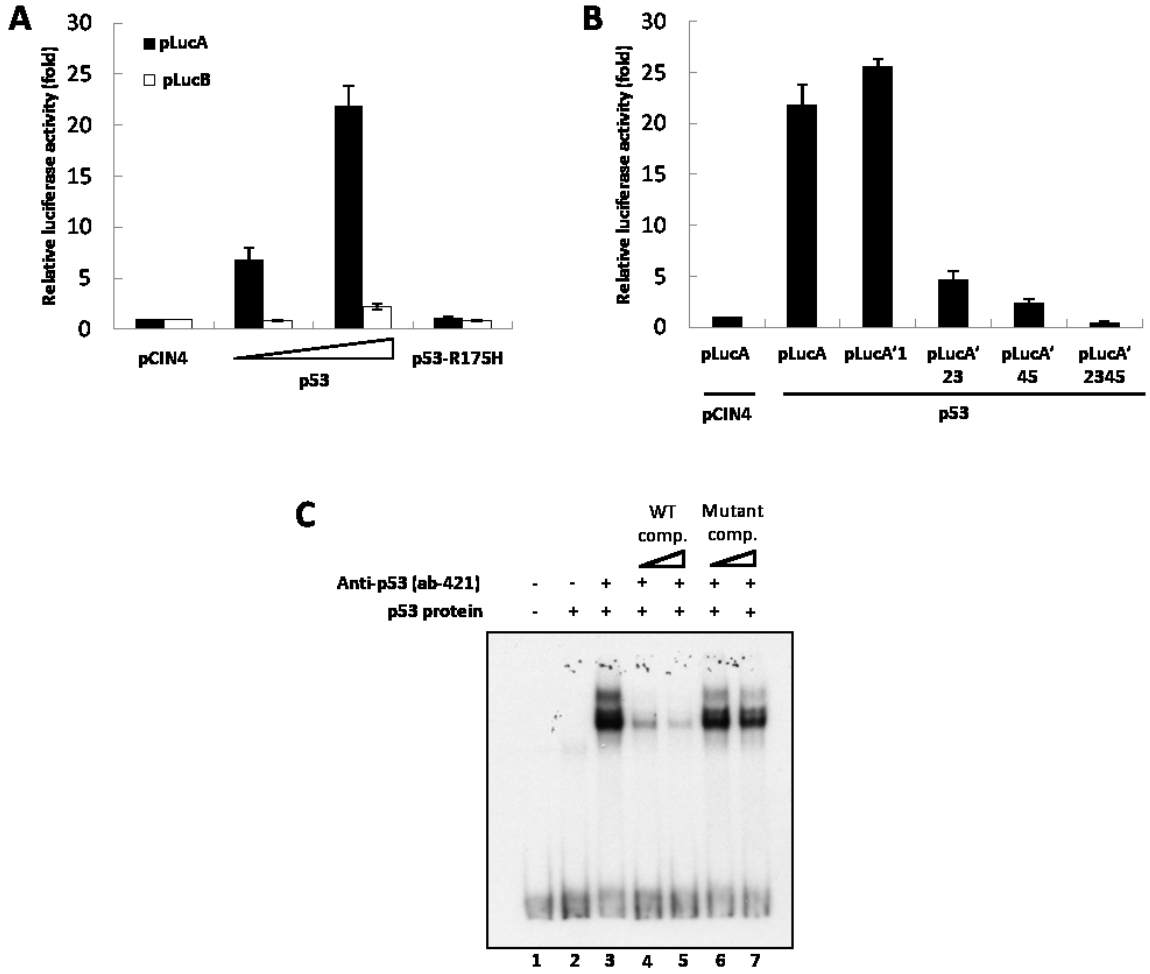

Figure 2.3. Promoter binding assays confirming p53-binding of *PANK1* promoter. **(A)** p53 activates luciferase activity of reporter construct containing p53-binding elements in the *PANK1* promoter. H1299 cells were transfected with control vector (pCIN4), increasing amounts of p53 expression vector, or vector expressing binding-deficient p53 mutant R175H along with luciferase constructs pLucA or pLucB for 24 hr before measuring luciferase activity. **(B)** Mutations of p53-binding elements on the reporter construct abolished p53-induced luciferase activity. H1299 cells were co-transfected with p53 expression vector and various mutated luciferase constructs for 24 hr before measuring luciferase activity. **(C)** Gel shift assay showing p53 binding on oligonucleotide containing p53-binding sites in the *PANK1* promoter region. The DNA binding activity of purified p53 protein was enhanced with C-terminal p53 antibody pAb421. Specificity of the binding was verified by competition with non-radiolabeled wild-type and mutant probes.

171-bp DNA fragment containing the four WT BHS, which was supershifted and enhanced in the presence of anti-p53 antibody (ab 421) (**Figure 2.3C**). Furthermore, binding of p53 to the radiolabeled fragment was outcompeted by the cold WT fragment, but not by the cold mutant fragment (**Figure 2.3C**). Together, these results indicate that the *PANK1* gene is a transcriptional target of p53 and that the two consensus p53-binding sites (four half-sites) at the 5'-end of *PANK1* exon 1α are responsible for p53-dependent *PANK1* gene activation.

p53 induces PanK1 expression

To confirm that *PANK1* is a p53-inducible gene, we examined *PANK1* mRNA and protein expression in response to p53 overexpression and DNA damage. Transfection of p53 expression vector in H1299 cells induced mRNA level of *PANK1α* as well as that of p21, but expression levels of *PANK1β*, *PANK2* and *PANK3* remain unchanged (**Figure 2.4A**). Similarly, PanK1α protein level increased in a p53 dose-dependent manner in H1299 cells transfected with p53 expression vector, but no induction was observed in the presence of mutant p53 R175H (**Figure 2.4B**). In HCT116 cells containing endogenous WT-p53, induction of PanK1α protein level was observed upon treatment with DNA-damaging agents (etoposide, actinomycin D and doxorubicin), which coincided with

Figure 2.4

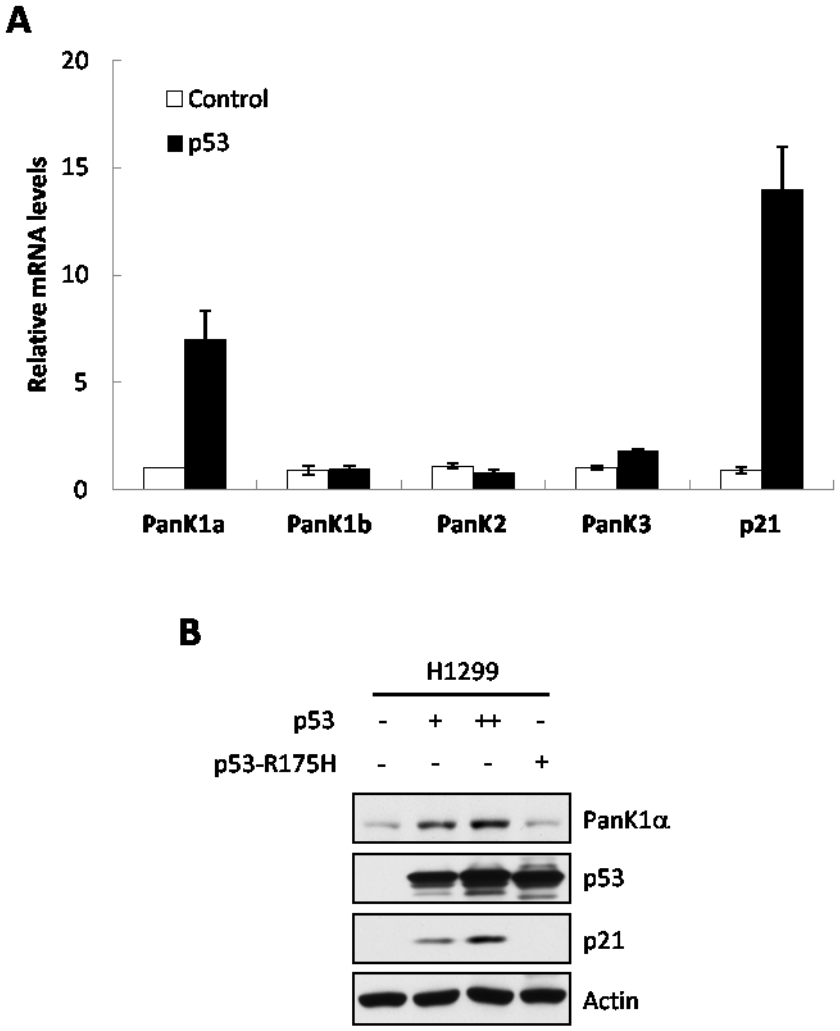

Figure 2.4. Overexpression of p53 protein induces PanK1 expression. (A) H1299 cells were transfected with either control or p53-expressing vectors, and mRNA were extracted from cells 24 hr after transfection and analyzed via RT-qPCR for PanK1α and p21 expressions. **(B)** Western blot analysis of the protein levels of PanK1α, p21, p53, and actin in H1299 cells transfected with control vector and vectors expressing wild-type and R175H mutant p53.

p53 induction. In contrast, PanK1α expression remained at the basal level in p53-deficient HCT116 cells, despite undergoing the same DNA-damaging treatments (**Figure 2.5A**).

We next investigate whether p53 can regulate PanK1β expression. Our PanK1 antibody (Bethyl) is able to detect exogenously expressed recombinant PanK1β isoform on Western blot, but our inability to detect endogenous PanK1β isoform in various cell lines tested is likely due to very low level expression of PanK1β. Moreover, PanK1β regulation by p53 could be cell-type specific, as suggested by the absence of PanK1β induction in H1299 cells in the presence of p53 (**Figure 2.4A**). In order to examine the possibility that p53 also regulates PanK1β expression, RT-qPCR was performed in HCT116 and U2OS cells treated with DNA damaging agents. Interestingly, PanK1β mRNA level was induced upon DNA damage in both cell lines, similar to the kinetics seen for PanK1α, while p53-deficient HCT116 cells fails to upregulate PanK1β expression (**Figure 2.5B** and **2.5C**). Together, our findings demonstrate that p53 upregulates the expression of both PanK1α and PanK1β *in vitro*, although regulation may be cell-type dependent.

Figure 2.5

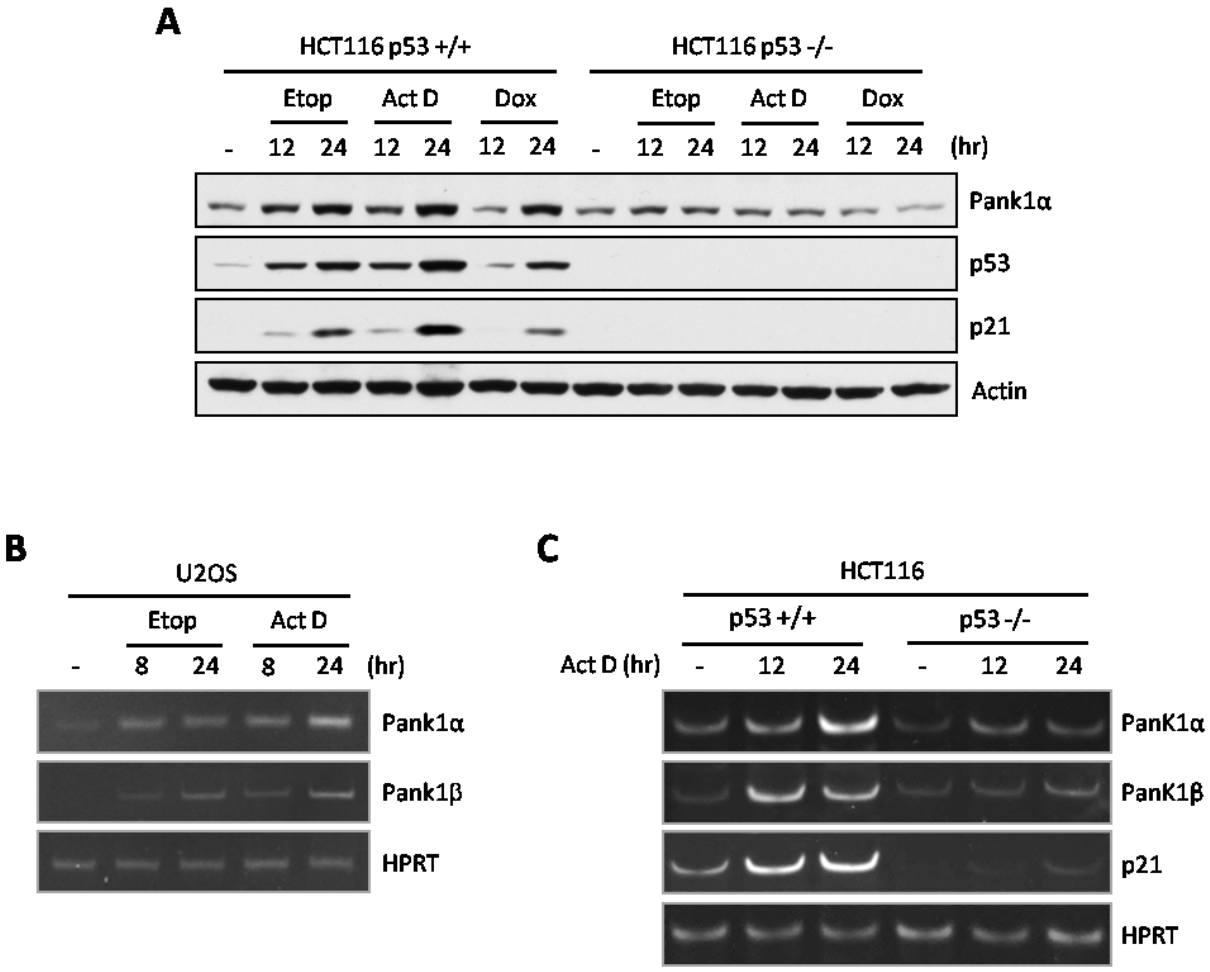

Figure 2.5. Endogenous p53 induces PanK1 expression. (A) HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with 20 μM Etoposide, 10 nM Actinomycin D, and 0.2 μg/mL Doxorubicin for 12 and 24 hrs, and cell lysates were analyzed by Western using PanK1, p53, p21 and actin antibodies. **(B)** U2OS cells were treated with 20 μM Etoposide and 10 nM Actinomycin D for 8 and 24 hrs, and mRNA levels of PanK1α and PanK1β were analyzed via RT-qPCR. **(C)** HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with 10 nM Actinomycin D for 12 and 24 hrs, and mRNA levels of PanK1α and PanK1β were analyzed via RT-qPCR.

PanK1 is not required for DNA damage-induced apoptosis

Since the major function of p53 is to act as a tumor suppressor that regulates cell fate under genotoxic stress, we wish to investigate whether PanK1 is a downstream modulator of p53-dependent cell cycle arrest or apoptosis. Depletion of PanK1 via siRNA in HCT116 cells containing WT-p53 did not have an effect on p21 induction or cleavage of proapoptotic effectors (caspase-3 and PARP) after actinomycin D treatment (**Figure 2.6A**). Similarly, PanK1 knockdown in HCT116 cells did not have an effect on cell death after DNA damage, as measured by the sub-G1 population of apoptotic cells using FACS analysis (**Figure 2.6B**). Together, these results demonstrate that while both α- and β-isoforms of PanK1 are induced by p53, PanK1 does not modulate the effect of p53-dependent genotoxic stress response.

p53 regulates PanK1 expression *in vitro* under starvation

The link between p53 and PanK1 suggests a role for p53 in CoA metabolism. As previously mentioned, regulation of CoA level in tissues is critical in response to various metabolic stresses in order to meet energy demands. Interestingly, p53 has been shown to modulate growth arrest and apoptosis under glucose starvation *in vitro*. Upon glucose withdrawal, p53 is activated by the energy-sensing AMPK that leads to downstream

Figure 2.6

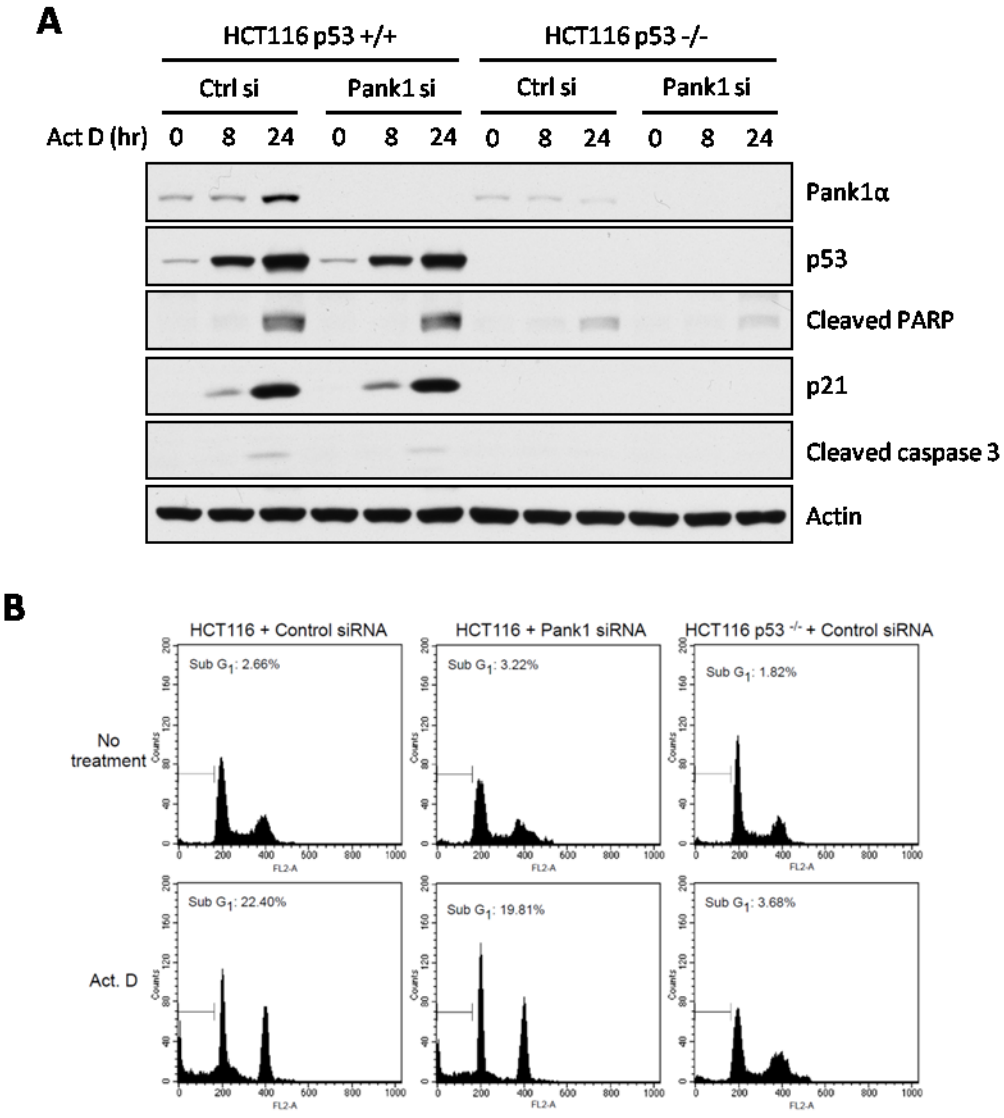

Figure 2.6. PanK1 is not required for DNA-damage-induced apoptosis. (A) HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with control of PanK1 siRNA for 36 hrs and were subsequently treated with 10 nM Actinomycin D for 0, 8 or 24 hrs. Total cell extracts were assayed for PanK1, p53, p21, cleaved PARP, cleaved caspase 3, and actin. **(B)** HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with control or PanK1 siRNA for 36 hrs and then treated with 10 nM Actinomycin D for 36 hrs. The cells were harvested, fixed with ice-cold methanol, and analyzed by flow cytometry. Cells with sub-G1 DNA content were scored as apoptotic cells.

expression of p53 targets [122,123]. Thus, it is possible that p53 regulates the expression of PanK1 during starvation.

To test this hypothesis, we subjected isogenic HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells to glucose starvation. Upon glucose depletion, p53 levels are induced in HCT 116 $p53^{+/+}$ cells and PanK1 expression remains unchanged. Surprisingly, PanK1 expression in HCT116 $p53^{-/-}$ cells decreased dramatically after glucose starvation in the absence of p53 upregulation (**Figure 2.7A**). To verify that p53 activity is indeed increased upon glucose starvation, we observed a steady increase in p53 downstream targets p21 and Puma as well as p53-induced apoptosis (cleaved caspase 3) in HCT116 $p53^{+/+}$ cells, but not in $p53^{-/-}$ cells.

p53 regulates PanK1 expression *in vivo* under starvation

The link between p53 and PanK1 suggests a role for p53 in CoA metabolism. As previously mentioned, regulation of CoA level in tissues is critical in response to various metabolic stresses in order to meet energy demands. Interestingly, p53 has been shown to modulate growth arrest and apoptosis under glucose starvation *in vitro*. Upon glucose withdrawal, p53 is activated by the energy-sensing AMPK that leads to downstream expression of p53 targets [122,123]. Thus, it is possible that p53 regulates the

Figure 2.7

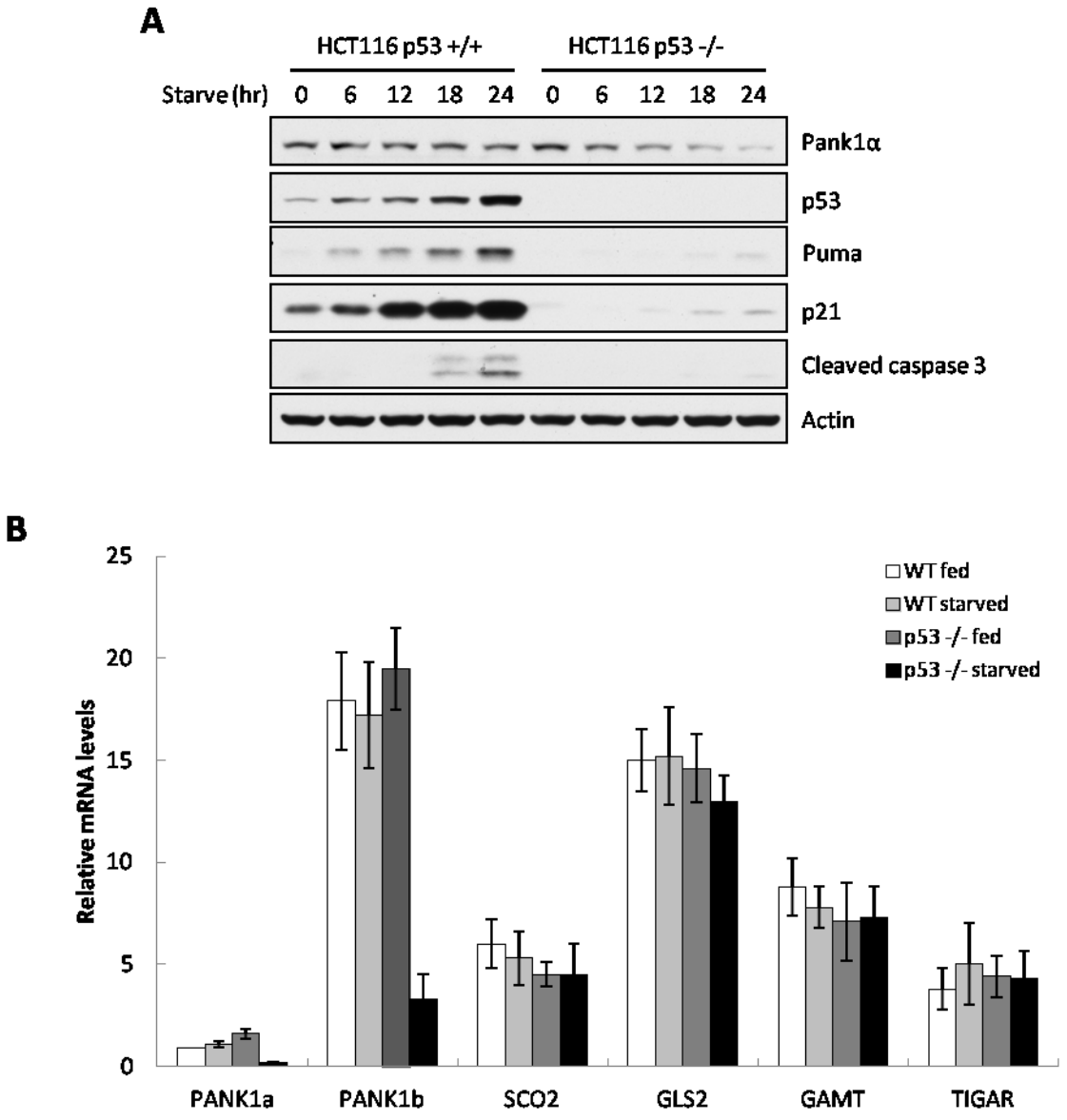

Figure 2.7. p53 regulates PanK1 expression *in vitro* and *in vivo* under starvation. **(A)** HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were glucose-starved (DMEM + 10% FBS + no glucose) for the times indicated. Total cell extracts were assayed for PanK1, p53, Puma, p21, cleaved caspase 3 and actin. **(B)** WT and $p53^{-/-}$ mice were either fed or starved for 3 days, and total RNA was extracted from the livers (WT fed, n = 3; WT starved, n = 3; $p53^{-/-}$ fed, n = 3; $p53^{-/-}$ starved, n = 3). RT-qPCR was performed to quantify the relative mRNA levels of PanK1 and other p53 metabolic targets.

expression of PanK1 during starvation.

PanK1, specifically the PanK1β isoform, is most highly expressed in the liver in both human and mouse. This is consistent with the fact that the liver contains the highest concentration of CoA and carries out most CoA-dependent anabolic and catabolic reactions. One of the major functions of the liver is to convert stored energy into usable fuel sources for different tissues and organs in the body during energy crises. Under long-term starvation, the liver undergoes gluconeogenesis from amino acid degradation and β-oxidation of fatty acids, both of which are processes that require CoA. With this in mind, we wish to examine whether p53 regulates PanK1 expression in the mouse liver during prolonged fasting. Since the p53 binding sites of *mPank1* is quite conserved compared to that of *hPANK1* (**Figure 2.1**), we assumed that the kinetics of mPanK1 expression under p53 control remains to a similar degree.

To determine the requirement of p53 on mPanK1β expression in the liver under nutrient deprivation, we subjected WT and p53-null mice to either fed or starved conditions for 3 days. The liver tissues were subsequently extracted, and the mRNA levels of mPanK1β were quantified. Under fed condition, expressions of mPanK1β were similar between WT and p53-null mice, suggesting that PanK1 expression is p53-independent under adequate energy intake (**Figure 2.7B**). However, after fasting,

mRNA levels of mPanK1β dropped significantly in the p53-null mice, while the WT mice was able to maintain the same basal levels of mPanK1β expression (**Figure 2.7B**). Our observation suggests that p53 is required for the maintenance of PanK1 expression in the liver during starvation. Interestingly, expression of other p53 metabolic targets did not change significantly in the liver with respect to both the presence of p53 and nutrient availability, implying that the expressions of these targets are p53-independent in the liver and do not respond to fasting (**Figure 2.7B**).

p53 regulates PanK1 activity and CoA levels in mice after fasting

Since p53 is required to maintain PanK1 expression during starvation, we wish to further investigate the functional consequence of PanK1 expression in the absence or presence of p53. First, we tested *in vitro* PanK activity of cell lysates from H1299 cells that were transfected with either p53 or control expression vector. In the presence of increasing amount of p53, PanK activity rises proportionally to p53 level (**Figure 2.8A**). We then tested PanK activity in the mouse liver of WT and p53-null mice that were subjected to either fed or starved conditions. Under fed condition, WT and p53-null mice exhibited similar levels in liver PanK activity, which is consistent with our findings that PanK1 expression is not p53-dependent under condition with excess nutrients (**Figure**

Figure 2.8

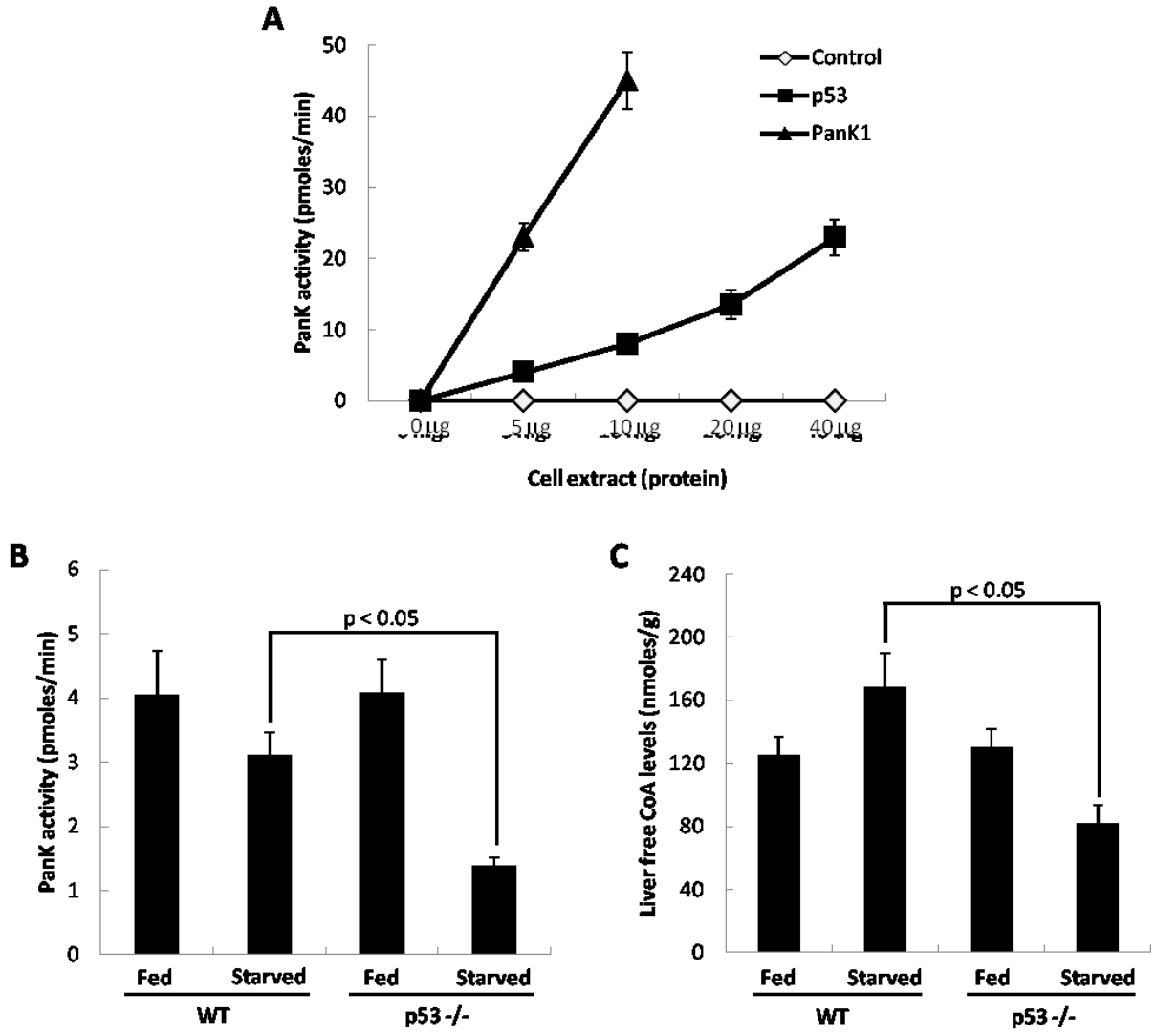

Figure 2.8. p53 regulates PanK1 activity and CoA levels *in vivo* after starvation. (A) H1299 cells were transfected with p53 or PanK1-expressing vectors, and total cell extracts were obtained to assay for PanK kinase activity *in vitro*. **(B)** WT and $p53^{-/-}$ mice were either fed or starved for 3 days, and total liver extracts were obtained and assayed for PanK activity (WT fed, n = 3; WT starved, n = 3; $p53^{-/-}$ fed, n = 3; $p53^{-/-}$ starved, n = 3). **(C)** Mice were treated as in B, and levels of liver free CoA were quantified according to the Experimental Procedures (WT fed, n = 3; WT starved, n = 3; $p53^{-/-}$ fed, n = 3; $p53^{-/-}$ starved, n = 3).

2.8B). Interestingly, p53-null mice exhibited significantly lower liver PanK activity compared to WT mice after 3-day starvation (**Figure 2.8B**), which is parallel to the dramatic decrease of PanK1 expression in the liver of starved p53-null mice shown before (**Figure 2.7B**). Furthermore, free CoA levels determined from the liver tissues correlate with the levels of PanK activity measured (**Figure 2.8C**). Together, these data suggest that p53 regulates CoA synthesis through maintenance of PanK1 expression during starvation.

p53 regulates fat metabolism and gluconeogenesis in the mouse liver

Since the availability of CoA is crucial for liver function during fasting, we wish to examine the consequences of the CoA level discrepancies observed between WT and p53-null mice. As previously mentioned, the liver takes up free fatty acids released from the fat cells and undergoes β-oxidation to generate ketone bodies, an important energy source for the heart and brain during fasting. β-oxidation of fatty acids requires free CoA, and we hypothesized that decreased synthesis of free CoA in fasting p53-null mice would compromise fatty acid degradation. Indeed, we observed increased concentration of long-chain acyl-CoA in the liver of fasting p53-null mice compared to control (**Figure 2.9A**). Similarly, histological staining of the liver tissues showed significantly greater

Figure 2.9

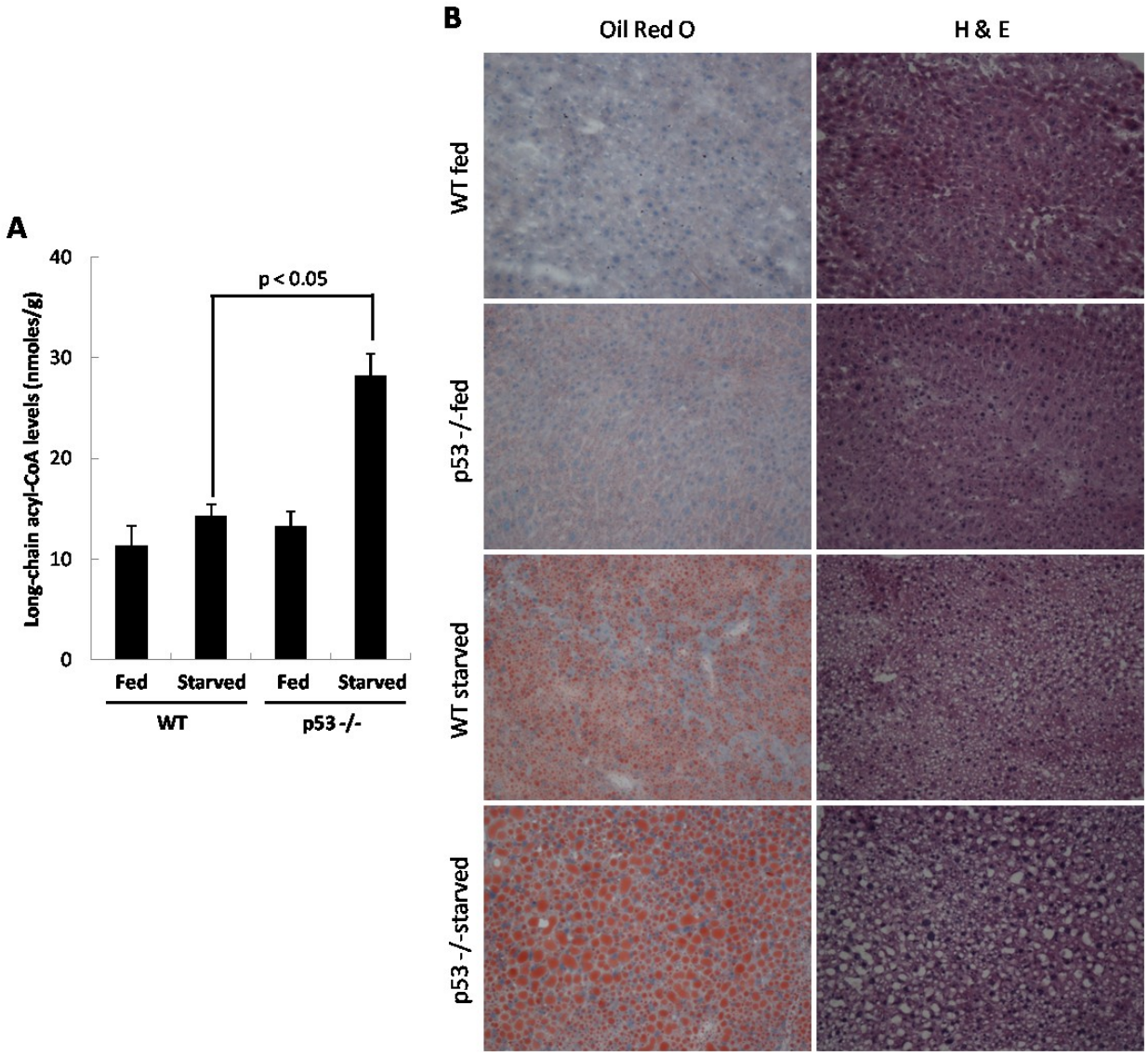

Figure 2.9. p53 regulates lipid metabolism in the mouse liver. (A) WT and $p53^{-/-}$ mice were either fed or starved for 3 days, and the levels of long-chain acyl-CoA in the liver were quantified according to the Experimental Procedures (WT fed, n = 3; WT starved, n = 3; $p53^{-/-}$ fed, n = 3; $p53^{-/-}$ starved, n = 3). **(B)** Liver sections obtained from WT and $p53^{-/-}$ mice either fed or starved for 3 days were stained with Oil Red O and H&E. The red staining from Oil Red O and the clear areas in the H&E staining indicate the presence of fat droplets in the liver tissue.

accumulation of fat droplets in the liver of starved p53-null mice compared to starved WT mice via Oil Red O staining (**Figure 2.9B**).

In addition to undergoing β-oxidation, the liver also carries out gluconeogenesis during fasting to provide energy for glucose-dependent tissues, such as red blood cells. There are two processes during gluconeogenesis that are CoA-dependent: 1) degradation of amino acid into TCA cycle intermediates; 2) activation of pyruvate carboxylase, the enzyme that catalyzes the first step of gluconeogenesis from pyruvate, by acetyl-CoA. We predicted that the shrinkage of free CoA pool would lead to decreased availability of glucogenic substrates derived from these processes, resulting in a slower rate of glucose production. As expected, fasting blood glucose levels were significantly lower in the p53-null mice, while no differences were observed for blood glucose levels between the two genotypes under normal feeding (**Figure 2.10A**). Furthermore, pyruvate challenge test performed after 16 hrs of fasting revealed a marked attenuation of gluconeogenic potential in the p53-null mice, as measured by their responding blood glucose levels after injection of sodium pyruvate (**Figure 2.10B**). Taken together, our findings demonstrate that CoA synthesis is p53-dependent under nutrient deprivation, and the decrease in liver free CoA levels in the absence of p53 results in the impairment of both fatty acid oxidation and gluconeogenesis.

Figure 2.10

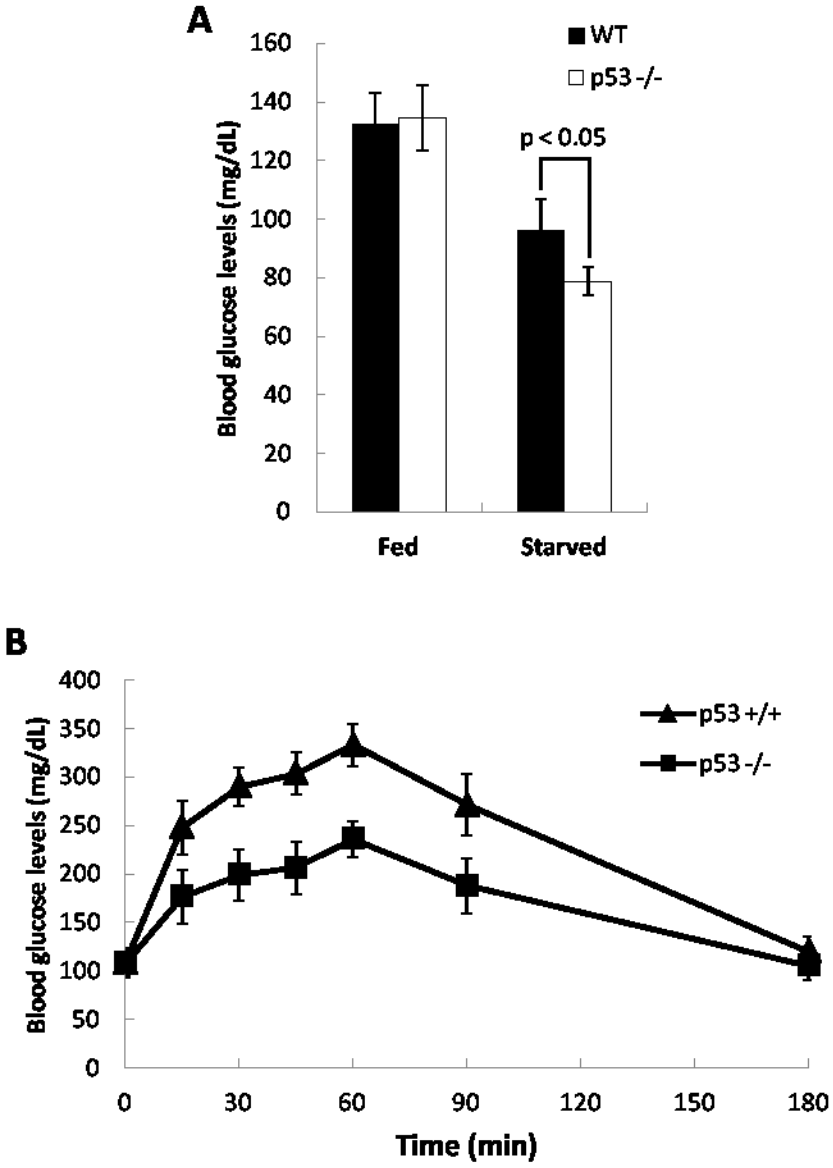

Figure 2.10. p53 regulates gluconeogenesis in the mouse liver. (A) Blood glucose levels were measured in WT and $p53^{-/-}$ mice that were either fed or starved for 2 days (WT fed, n = 4; WT starved, n = 4; $p53^{-/-}$ fed, n = 5; $p53^{-/-}$ starved, n = 5). **(B)** Pyruvate challenge test was performed using WT and $p53^{-/-}$ mice that were starved for 16 hours (WT mice, n = 5; $p53^{-/-}$ mice, n = 7). Blood glucose levels were measured at different time points after intra-peritoneal injection of sodium pyruvate.

In summary, we have identified PanK1 as a p53 metabolic target and uncovered a novel role of p53 in regulating liver CoA abundance during nutrient deprivation through PanK1 expression. The involvement of p53 in the physiological response to starvation provides clues to the function of p53 in the liver and highlights the significance of p53 function in homeostasis.

2.2. Chapter 2: Identification of a novel p53 acetylation site at lysine residue K101

2.2.1. Background and Rationale:

Recently, roles of p53 have expanded beyond the canonical function of apoptosis and cell growth arrest, and now include cellular processes such as metabolism, senescence, aging and autophagy. Because of the extraordinary diversity of p53 functions, many have postulated that mechanisms exist to allow p53 to selectively activate downstream targets in specific functional groups, depending on the cellular context.

p53 activity is regulated by a complex network of fine-tuning mechanisms that include p53 protein stability, co-activator recruitment, and a diverse array of post-translational modifications, including acetylation, ubiquitination, phosphorylation, methylation, sumoylation, and neddylation (see Section 1.3). Specifically, acetylation of p53 has been established to play a major role in controlling promoter-specific activation of downstream targets during stress responses. Our lab has demonstrated that acetylation at K120 by Tip60/MOF is important for p53-mediated apoptosis, while acetylation at K164 by CBP/p300, along with K120 acetylation, contributes to p53-induced cell cycle arrest [93,95]. More recently, the 3KR mouse model that expresses acetylation-deficient p53 (K117/161/162R), which mirrors the K120/164R

mutations in human p53, demonstrated that while apoptotic and growth arrest functions of p53 are lost, p53-dependent metabolic regulation is still intact [107]. These studies suggest that there are potentially a large number of p53 targets that can still be transcriptionally activated in the absence of K120/164 acetylation (K117/161/162R in mouse), including targets such as MDM2, TIGAR and GLS2. Moreover, additional modifications may exist that are crucial for the regulation of p53 targets other than pro-apoptotic and growth arrest genes.

With this in mind, we conducted a screen using mass spectrometry to uncover previously unknown modifications. Here, we identified a novel p53 acetylation site at lysine K101 within the DNA-binding domain. Lysine residue K101 of p53 is evolutionarily conserved and has been found to be mutated in human cancers. In this study, we demonstrated that K101 in human p53, as well as the homologous K98 lysine residue in mouse p53, can be acetylated by acetyltransferase CBP. Acetylation at this novel site does not contribute to p53 stability or DNA-binding capabilities. Ablation of K98 acetylation in mouse p53 alone does not affect the transcriptional activity of p53. However, simultaneous loss of K98 acetylation with the previously characterized K117/161/162 acetylations significantly abrogates p53-mediated activation of TIGAR and MDM2 genes.

2.2.2. Materials and Methods:

Cell Culture and Transfection. H1299 cells were maintained in DMEM and HCT116 cells in McCoy's 5A medium. All media were supplemented with 10% fetal bovine serum. Transfections with plasmid DNA were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Tet-on inducible H1299 stable lines were generated by transfecting pTRIPZ tetracycline-inducible constructs into H1299 cells, followed by puromycin selection (1 μg/mL) for 14 days. Established Tet-on H1299 stable lines were maintained in DMEM supplemented with 10% Tet-free FBS (Clonetech).

Plasmids and Antibodies. The plasmids expressing various p53 mutants were derived from pCin4-Flag-p53 by mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instuctions. Mutations at the specific sites were confirmed by DNA sequencing. Tet-on inducible mouse p53-expressing constructs were generated by subcloning Flag-p53 fragments from pCin4-Flag-p53 into pTRIPZ tetracycline-inducible vector (Thermo). TIGAR luciferase construct was generated by subcloning a region of the TIGAR promoter containing a p53 binding site into pBVLuc luciferase reporter vector (He et al., 1998) using forward primer 5' – GGCTATCGAGGGAAGGAATC – 3' and reverse primer 5' –

AGGGGGAACCTCAGAAACTG – 3'. Rabbit monoclonal site-specific antibody against acetylated K101 p53 was generated in collaboration with Epitomics/Abcam Inc.

Protein Purification and Mass Spectrometry. To purify the acetylated p53 protein for mass spectrometric analysis, H1299 cells were co-transfected with CMV-Flag-p53 and CMV-CBP-HA, cultured for 16 hours, and then treated with 1 μM TSA + 5 mM Nicotinamide for 8 hours. Cells were harvested and lysed in the Flag-lysis buffer (50 mM Tris-HCl pH 7.9, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, and fresh proteinase inhibitor cocktail (Sigma)) plus 2 μM TSA and 10 mM Nicotinamide. The cell extracts were then immunoprecipitated with the anti-Flag monoclonal antibody-conjugated M2 agarose beads (Sigma), and eluted using Flag peptide (Sigma). The eluted material was resolved by SDS-PAGE on a 4-20% Tris-Glycine gradient gel (Invitrogen), and the p53 bands were excised and subjected to mass spectrometric analysis.

Immunoprecipitation and Immunoblot. To immunoprecipitate ectopically expressed Flag-p53 proteins, cells were lysed in the Flag-lysis buffer (50 mM Tris-HCl [pH 7.9], 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% sarkosyl, 10% glycerol, and

fresh proteinase inhibitor cocktail). Whole cell extracts were immunoprecipitated with the anti-Flag monoclonal antibody-conjugated M2 agarose beads (Sigma). The immunoprecipitated products were eluted with Flag peptide (Sigma), resolved by SDS-PAGE, and detected by antibodies as indicated.

To detect acetylation of the endogenous p53 proteins at K101, HCT116 cells were lysed in Flag lysis buffer. Five milligrams of total cell extracts were incubated with 1 μg of the site-specific antibody against acetylated p53 at K101 overnight with rotation. Twenty microliters of protein A agarose beads were then added and incubated for 2 hours. Beads were washed five times with Flag lysis buffer, and the bound materials were eluted in the SDS sample buffer with boiling. The eluted materials were resolved on SDS-PAGE gel and detected by the anti-p53 DO-1 antibody (Santa Cruz).

RNA Extraction and RT-qPCR. Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse Transcriptase kit (NEB). PCR analysis was carried out using Applied Biosystems 7500 Fast System with the following primers: 5' – ctcaagacttcgggaaagga – 3' and 5' – ggtgtaaacacagggcactctt – 3' for TIGAR expression.

Luciferase Activity Assay. Transfection of H1299 cells were performed in 24-well plate using 0.2 μg TIGAR luciferase reporter constructs, 0.05 μg p^{RL}-tk Renilla construct, and various amount of vectors expressing wild-type or mutant p53. Luciferase activities were measured 24 hours post-transfection using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized with Renilla luciferase activities to obtain the relative luciferase activity.

Chromatin Immunoprecipitation (ChIP). The procedure for this assay is essentially described in Section 2.1.2 Materials and Methods. The purified DNA were then analyzed by PCR within linear amplification range followed by agarose gel electrophoresis. The following PCR primers were used: 5' – CGGCAGGTCTTAGATAGCTT – 3' and 5' – GGCAGCCGGCATCAAAAACA – 3' for TIGAR; 5' – GCACTCTTGTCCCCCAGGCT – 3' and 5' – GGTCTCCTGTCTCCTACCAT – 3' for p21.

2.2.3. Results and Discussion:

p53 is acetylated at the K101 lysine residue by CBP

To screen for unidentified acetylation of p53, we purified and analyzed ectopically expressed p53 protein in H1299 cells in the presence of different histone

acetyltransferases by mass spectrometry. To increase the yield of acetylated forms of p53, we treated the cells with deacetylases inhibitors trichostatin A (TSA) and nicotinamide for 8 hours before isolating the acetylated p53. Our mass spectrometry data showed that in the presence of acetyltransferase CBP, p53 is found to be acetylated at lysine residue K101, a previously uncharacterized p53 modification (**Figure 2.11A** and **2.11B**). The K101 lysine residue reside in the DNA-binding domain of p53, and is homologous to the K98 lysine residue in mouse p53 (**Figure 2.11A**). Interestingly, the K101 lysine residue appears to be evolutionarily conserved (**Figure 2.11B**), and has been found to be mutated in several different human malignancies.

To validate the acetylation at K101, we generated a rabbit monoclonal antibody specifically against p53 acetylated at lysine K101 (see 2.2.2 Materials and Methods). The specificity of the antibody for Western blot was tested by comparing the signal detected between wild-type p53 and acetylation-deficient p53 mutant with the K101 lysine residue replaced by arginine (K101R p53). H1299 p53-null cells were transfected with either Flag-tagged wild-type p53 or K101R p53 in the absence or presence of acetyltransferase CBP, and the tagged p53 were immunoprecipitated and resolved on SDS-PAGE for Western blot analysis. The site-specific AcK101-p53 antibody only recognized wild-type p53 acetylated by CBP, but not acetylation-deficient K101R p53

Figure 2.11

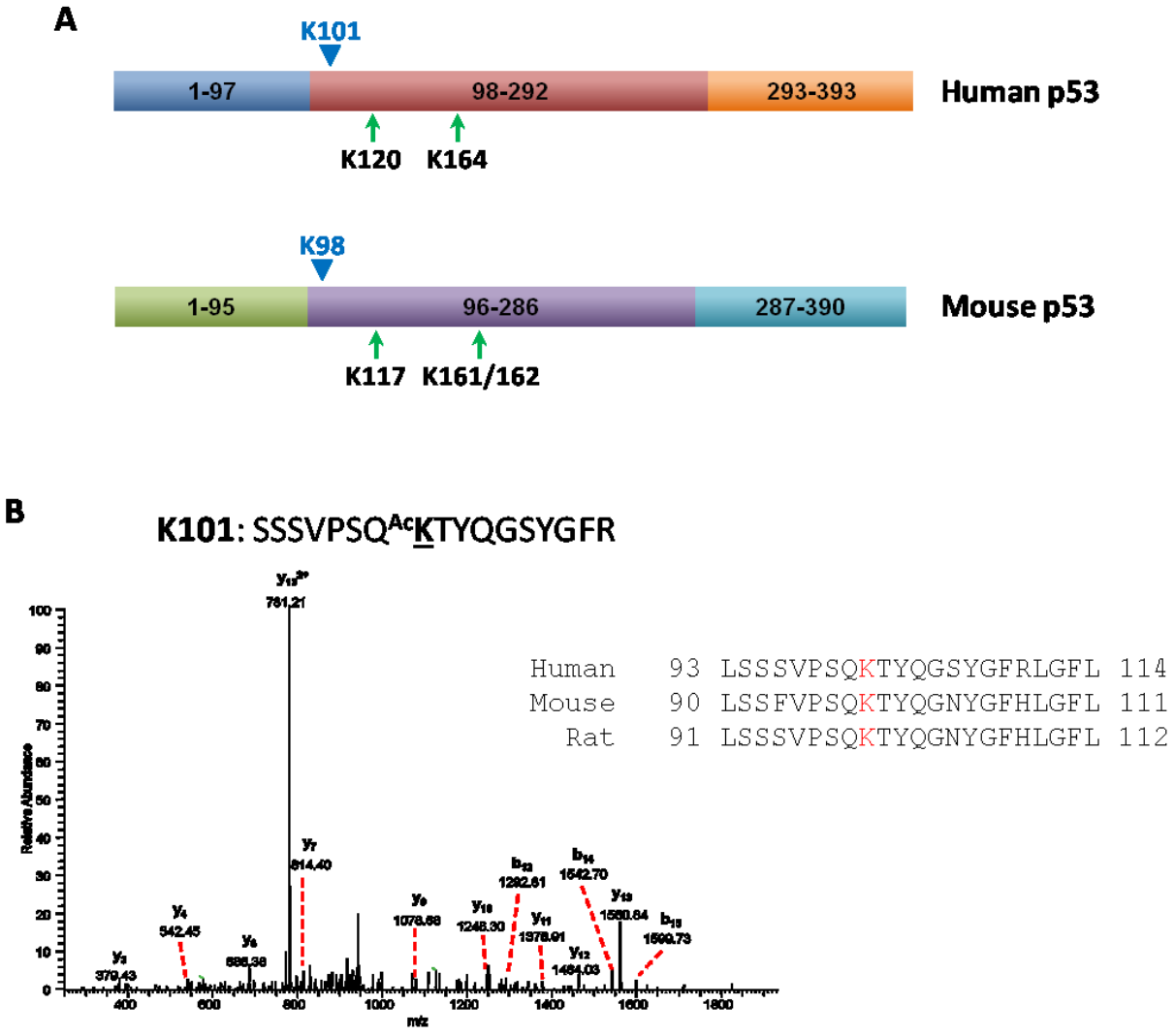

Figure 2.11. Identification of novel p53 acetylation site at lysine K101. (A) Cartoon figure depicting the K101 lysine residue located in the N-terminal end of the DNA-binding domain. The K98 lysine residue in the mouse p53 is homologous to the K101 lysine in the human p53. **(B)** Mass spectrometry of purified p53 protein in the presence of CBP showed acetylation of p53 at the K101 lysine residue. Cross species alignment of amino acids showed that the K101 lysine residue is evolutionarily conserved.

mutant (**Figure 2.12A**). Furthermore, K101-acetylated form of p53 was only detected in the presence of CBP, which confirmed the mass spectrometry data that CBP indeed catalyzes the acetylation of p53 at K101. Similar experiment was performed using mouse p53, and the AcK101-p53 antibody also recognized acetylation of mouse p53 at K98 in the presence of CBP (**Figure 2.12B**). Next, we wish to investigate whether p53 acetylation at K101 occurs endogenously. AcK101-p53 antibody was used to immunoprecipitate acetylated form of p53 in HCT116 cells after DNA damage (Actinomycin D, Etoposide and Doxorubicin), and the levels of AcK101-p53 were detected using anti-p53 DO-1 antibody. As seen in **Figures 2.13A** and **2.13B**, the steady-state levels of AcK101-p53 increased as p53 became stabilized after drug treatments. These results indicate that K101 of p53 is acetylated both *in vitro* and *in vivo*.

Simultaneous mutations at K117/161/162 and K98 impair p53 transcriptional activities

Previous studies have shown that ablation of acetylation at human K120 or mouse K117 alone impairs p53-mediated apoptotic induction, while ablation of human K120/164 or mouse K117/161/162 abrogates the ability of p53 to transactivate both growth arrest and apoptotic genes. Interestingly, 3KR p53 with triple lysine-to-arginine mutations

Figure 2.12

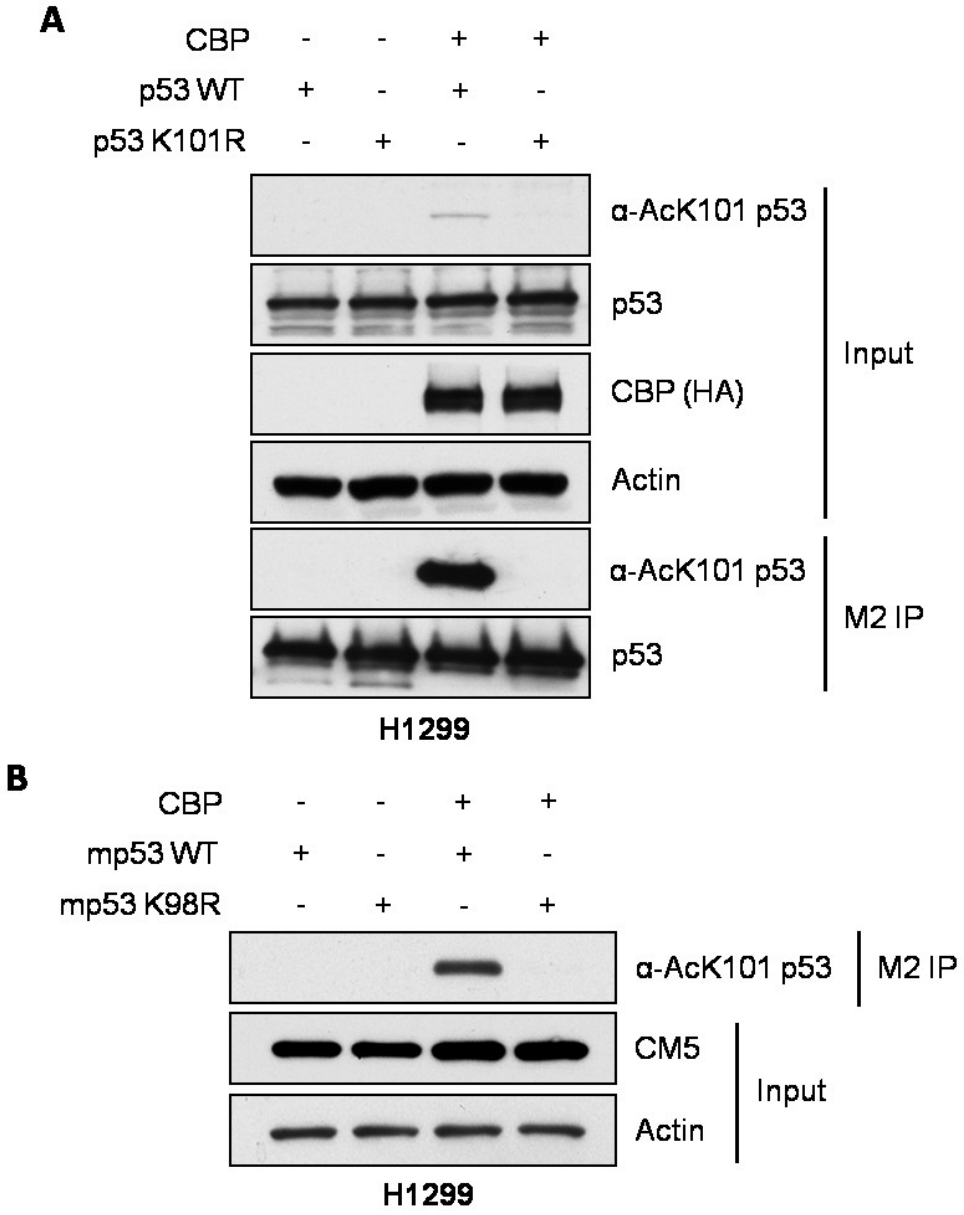

Figure 2.12. p53 is acetylated by CBP at lysine K101. (A) H1299 cells were transfected with vectors expressing either Flag-tagged human wild-type or K101R mutant p53 in the presence or absence of CBP. Whole cell lysates were collected and Flag-tagged p53 were immunoprecipitated using M2 Flag beads. Whole cell lysate and immunoprecipitated products were immunoblotted with site-specific antibody against acetylated p53 at K101. **(B)** Similar experiment to (A) was performed using Flag-tagged mouse wild-type or K98R mutant p53, and p53 acetylation at K98 was detected using the same site-specific antibody against acetylated K101.

Figure 2.13

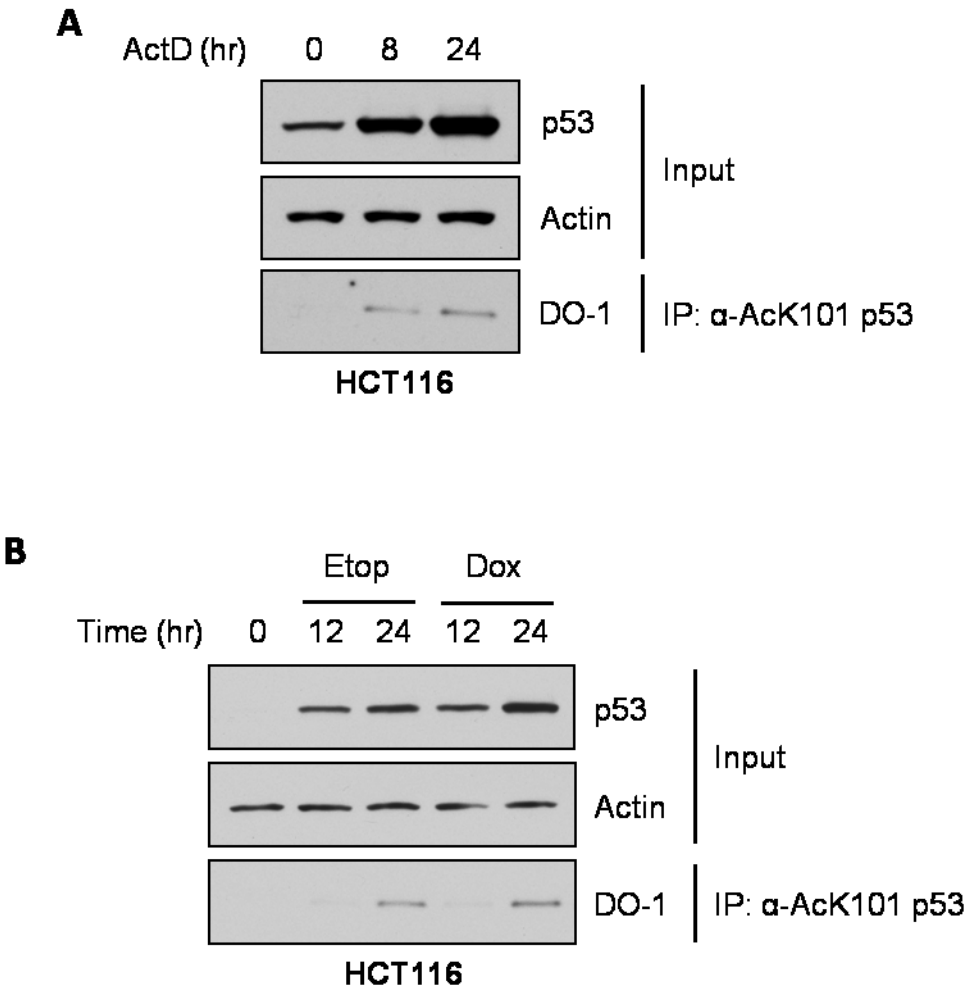

Figure 2.13. p53 is acetylated at K101 endogenously after DNA damage. (A) HCT116 cells were treated with 10 nM Actinomycin D for 0, 8 and 24 hours. K101-acetylated fractions of p53 were immunoprecipitated using site-specific antibody from whole cell lysates, and the immunoprecipitated products were detected using DO-1 (p53) antibody. **(B)** Similar experiment to (A) was performed using 20 μM Etoposide and 0.2 μg/mL Doxorubicin treatments for 0, 12 and 24 hours.

K117/161/162R retains regulation on Mdm2 and certain metabolic targets. In light of those findings, we wish to investigate whether ablation of K98 acetylation would further impair p53 transcriptional activities. Ectopic expression of K98R p53 in H1299 cells showed that the transcriptional activity of this single-mutation p53 was comparable to wild-type p53. However, when simultaneously mutating both 3KR (K117/161/162R) and K98R, the resulting 4KR98 p53 mutant exhibit significant defect in transactivating Mdm2 and Tigar expressions (**Figure 2.14**).

To characterize the dynamics of downstream target expression in a more physiological manner, Tet-on inducible H1299 stable lines conditionally expressing wild-type p53 and various p53 mutants were generated. Induction of wild-type p53 and 3KR p53 expression by doxycycline treatment led to increased expression of Mdm2 and Tigar, while induction of the 4KR98 mutant p53 failed to do so (**Figure 2.15A** and **2.15B**). These findings demonstrate that ablation of the K98 acetylation alone does not affect p53 activity, suggesting functional redundancy through other acetylations (such as acetylations at K117/161/162). However, disrupting all four acetylation sites exhibit significant defect in p53 transcriptional activity, indicating that in the absence of K117/161/162 acetylations, K98 acetylation may be critical for p53-mediated regulation on Mdm2 and metabolic targets.

Figure 2.14

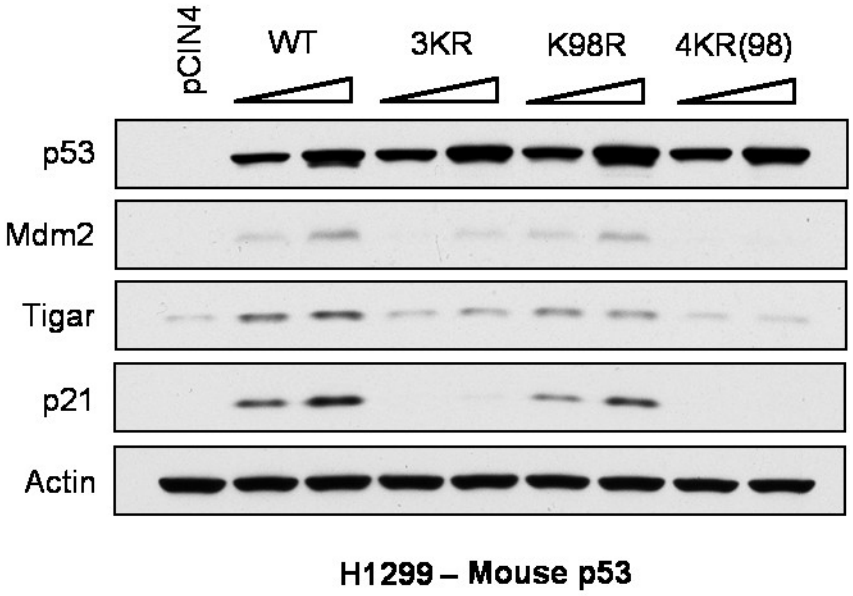

Figure 2.14. Effect of K98 acetylation on the activation of p53 targets in overexpression system. H1299 cells were transfected with empty vector and vectors expressing wild-type and various KR mutant p53. Expressions of p53 downstream targets were detected via Western blotting using antibodies against Mdm2, Tigar, and p21.

Figure 2.15

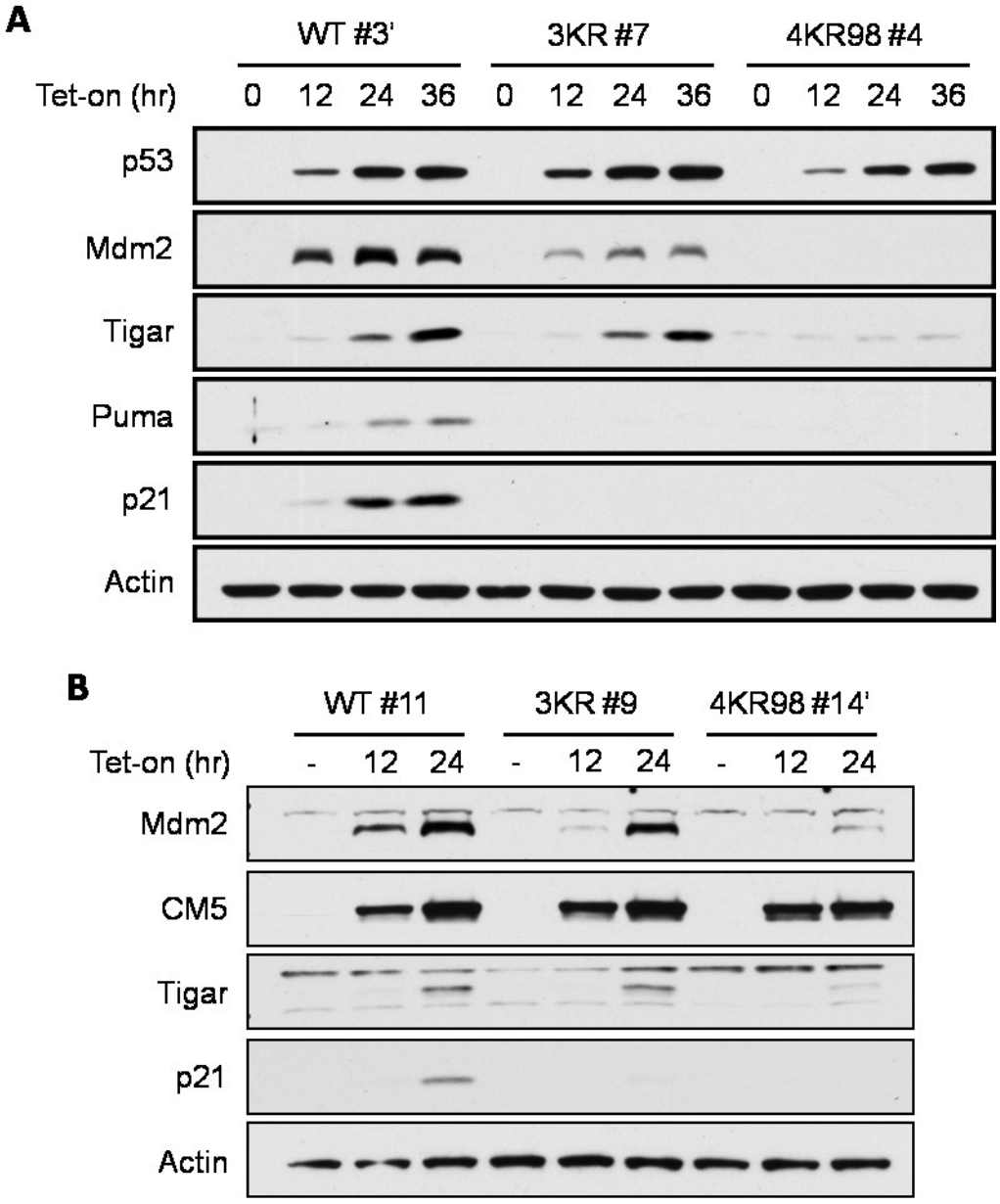

Figure 2.15. Effect of K98 acetylation on the activation of p53 targets in Tet-on system. (A) H1299 inducible-p53 stable lines expressing wild-type p53 or 3KR/4KR98 acetylation-deficient p53 were treated with 5 μg/mL doxycycline for 0, 12, 24 and 36 hours to induce p53 expression. Protein expressions of p53 targets were detected via Western blotting using Mdm2, Tigar, Puma and p21 antibodies. **(B)** Additional set of H1299 inducible-p53 stable lines were established expressing wild-type, 3KR or 4KR98 p53, and downstream p53 targets were analyzed as (A).

To confirm that the defect in activating downstream targets by 4KR98 p53 is transcriptional in nature, we quantified the mRNA levels of TIGAR after p53 induction in Tet-on inducible stable lines. Indeed, induction of wild-type and 3KR p53 enhanced TIGAR mRNA expression, while induction of 4KR98 p53 failed to do so (**Figure 2.16A**). Similarly, co-transfection of TIGAR luciferase reporter construct with vector expressing 4KR98 p53 mutant in H1299 resulted in diminished reporter activity, compared to transfection of vectors expressing wild-type and 3KR mutant (**Figure 2.16B**).

Acetylation in the DNA-binding domain does not affect p53 stability or DNA binding

One of the functions of p53 acetylation is to increase p53 stability. C-terminal lysine residues of p53 have been shown to be ubiquitinated that leads to p53 degradation, and that acetylation can occupy these lysine residues to prevent ubiquitination, which thereby, increases the stability of p53. Therefore, we wish to examine whether or not human K101/mouse K98 and other acetylation sites in the DNA-binding domain affect p53 stability. We observed that in the presence of Mdm2, wild-type p53 and the lysine-to-arginine mutants are degraded to a similar degree (**Figure 2.17A**). Similar results were obtained using mouse p53, suggesting that acetylation in the DNA-binding

Figure 2.16

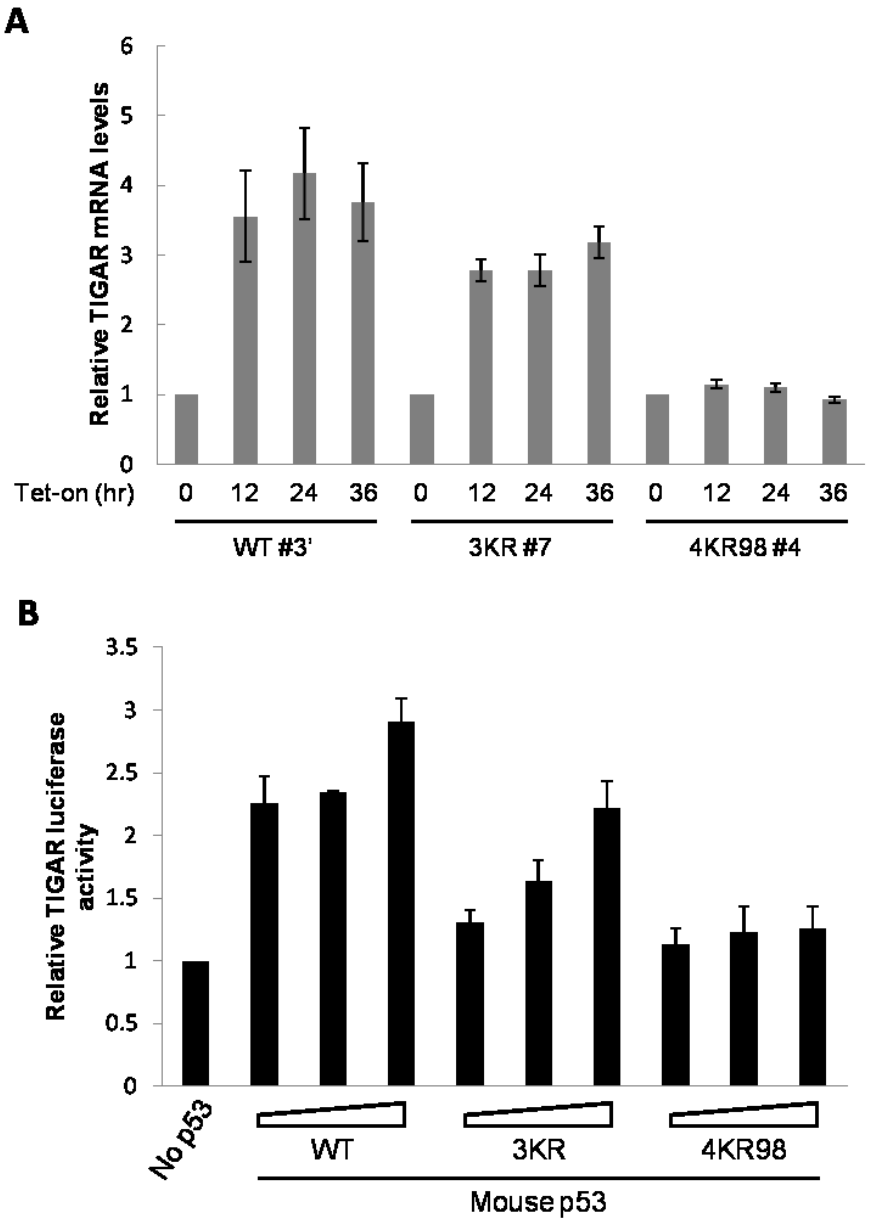

Figure 2.16. K98 acetylation affects transcription of TIGAR gene. (A) H1299 inducible-p53 stable lines expressing wild-type, 3KR or 4KR98 p53 were induced with 5 μg/mL doxycycline for 0, 12, 24 and 36 hours, and total RNA were extracted. Subsequently, reverse transcription was performed on extracted RNA to obtain cDNA, and quantitative PCR was performed to measure TIGAR mRNA levels. **(B)** H1299 cells were transfected with TIGAR luciferase construct along with increasing amount of plasmids expressing wild-type, 3KR or 4KR98 p53. Cell lysates were collected and assayed for luciferase activity.

Figure 2.17

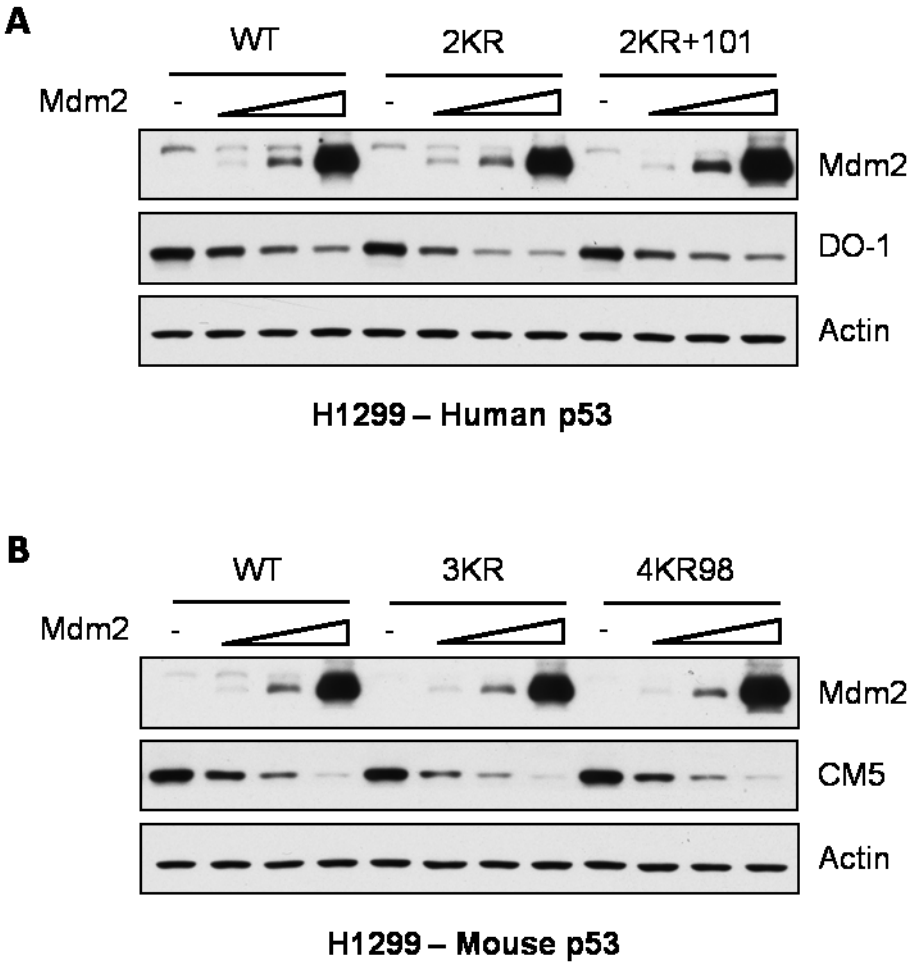

Figure 2.17. Acetylation in the DNA binding domain does not affect p53 stability. H1299 cells were transfected with vectors expressing either **(A)** human p53, or **(B)** mouse p53 in the presence of increasing amount of Mdm2-expressing plasmid. The levels of Mdm2 and p53 proteins were determined via Western blotting using Mdm2 and DO-1 (human p53) or CM5 (mouse p53) antibodies.

domain does not affect p53 stability (**Figure 2.17B**).

Acetylation in the DNA-binding domain also raises the question of whether these acetylations alter the affinity of p53 to DNA. To assess the DNA binding capabilities of p53 with different acetylation potentials, we performed chromatin-immunoprecipitation (ChIP) using wild-type and various acetylation-deficient p53. After doxycycline-induced p53 expression in the Tet-on inducible cell lines, wild-type p53, as well as 3KR and 4KR98 p53, was able to recruit to both p21 and TIGAR promoters (**Figure 2.18**). Collectively, these data suggest that acetylation in the DNA-binding domain does not affect p53 stability or DNA binding. Therefore, the mechanism underlying the transcriptional defects observed for both 3KR and 4KR98 p53 remain to be elucidated.

Figure 2.18

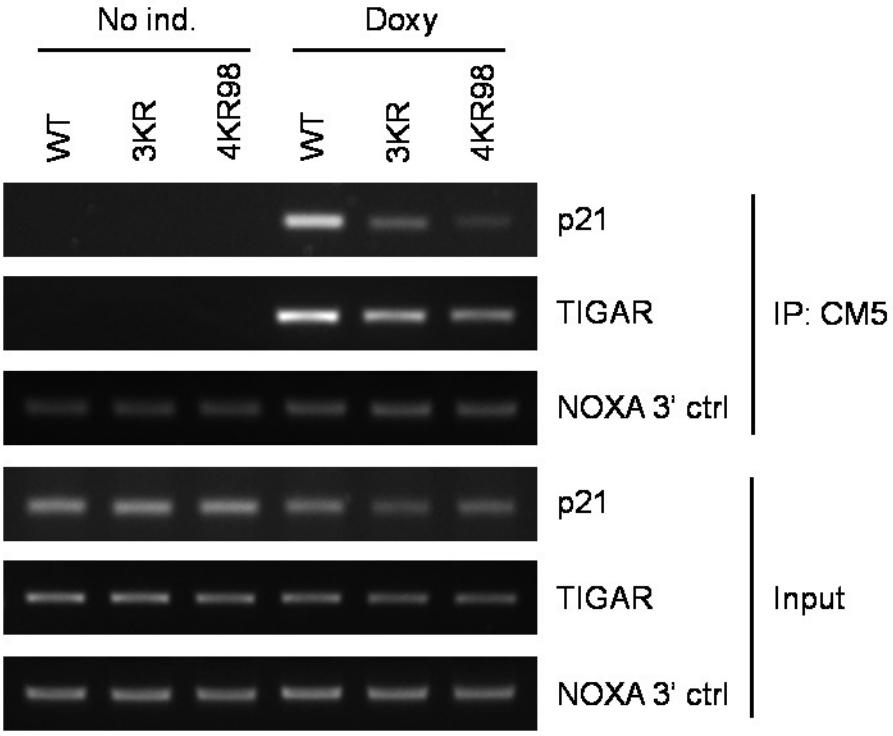

Figure 2.18. Acetylation-deficient p53 mutants retain the ability to bind p21 and TIGAR promoters. ChIP assay was performed on Tet-on inducible H1299 cells conditionally expressing wild-type, 3KR, and 4KR98 p53 that were treated with doxycycline to induce p53 expression for 8 hours. The immunoprecipitated DNA were amplified by PCR using primers that span the p53-binding region on p21 and TIGAR promoters, and the PCR products were resolved on agarose gel.

2.3. Chapter 3: TNFRSF14 is a novel non-canonical p53 target that contributes to tumor suppression

2.3.1. Background and Rationale:

Mechanism of how p53 confer tumor suppression is still unclear. While apoptotic and growth arrest functions of p53 can obviously halt or eliminate cells exposed to genotoxic stress or oncogenic activation, several evidence indicate that these functions of p53 are not essential for suppressing spontaneous tumor formation (see Section 1.4). These findings shifted focus towards the importance of non-canonical functions of p53 in tumor suppression, with emphasis on p53 metabolic regulation for some time. However, to complicate the p53 tumor suppression model even further, many metabolic targets of p53 may play dual opposing roles of preventing cancerous metabolic alterations on the one hand, and tumor survival on the other (see Section 1.4.2). In light of these collective observations, we set forth to identify novel p53 targets that can contribute to p53-mediated tumor suppression. Specifically, we wish to uncover targets that are still regulated by the 3KR p53 acetylation-deficient mutant, since the 3KR p53 mutant still retain tumor suppressive activities (see Section 2.2).

From a RNA-seq expression profiling on Tet-on inducible cell line expressing 3KR p53, we identified TNFRSF14 (tumor necrosis factor receptor superfamily, member 14)

as a novel p53 downstream activation target. TNFRSF14 is a 283-amino acid transmembrane glycoprotein that belongs to the tumor necrosis factor receptor (TNFR) family. Also known as HVEM (herpesvirus entry mediator), TNFRSF14 was initially discovered as a cell-surface mediator for HSV infection [128]. Unlike many members in the TNFR family, this TNFRSF14 does not contain a cytoplasmic death domain. Instead, TNFRSF14 is stimulated by its canonical TNF-related cytokine ligand LIGHT (TNFSF14/HVEM-L) to activate downstream NF-κB pathway during T-cell activation [129]. While most functional studies on TNFRSF14 demonstrated its role in T-lymphocyte activation and crosstalk between lymphoid cells, evidence also showed that this receptor, surprisingly, also play a role in cancer. Cells such as HT-29 and MDA-MB-231 that express TNFRSF14 receptor exhibit inhibition of cell growth or apoptosis in the presence of LIGHT ligand [130,131]. Hematological malignant cells with abundant TNFRSF14 expression can also be sensitized by LIGHT to induce cell death [132,133]. Strikingly, several independent cohort studies observed that the TNFRSF14 gene is frequently mutated in follicular lymphomas and diffuse large B-cell lymphomas [134-137].

Here, we report that TNFRSF14 is a novel p53 target that can be activated by 3KR p53. Interestingly, 4KR98 p53 mutant exhibit significant defect in transactivating the

TNFRSF14 gene. LIGHT ligand expression stimulates cell death in TNFRSF14-expressing cells. Moreover, LIGHT expression triggers cell death in Tet-on inducible cell line expressing 3KR p53, but not in those expressing 4KR98 p53.

2.3.2. Materials and Methods:

Cell Culture and Transfection. H1299, Saos2 and MCF7 cells were maintained in DMEM and HCT116 and HT29 cells in McCoy's 5A medium. All media were supplemented with 10% fetal bovine serum. Established Tet-on H1299 stable lines were maintained in DMEM supplemented with 10% Tet-free FBS (Clonetech). Transfections with plasmid DNA were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol.

Plasmids. Vector expressing full-length LIGHT ligand (TOPO-LIGHT) was generated by amplifying the cDNA and performing the TA-cloning into the TOPO vector (Invitrogen). Vector expressing secreted soluble LIGHT ligand (CMV9-sLIGHT) was generated by amplifying the cDNA and cloning into the Flag-CMV9 vector (Sigma). Tet-on inducible TNFRSF14-expressing construct was generated by subcloning TNFRSF14 cDNA fragment into pTRIPZ tetracycline-inducible vector (Thermo). All cDNA clones were

purchased from Thermo Scientific mammalian ORF collections. The following primers were used to amplify the various cDNAs: 5’ – ATGGAGGAGAGTGTCGTACGG – 3’ and 5’ – TCACACCATGAAAGCCCCG – 3’ for TOPO-LIGHT; 5’ – AGTCAAGCTTCAAGAGCGAAGGTCTCACGAG – 3’ and 5’ – AGTCGAATTCTCACACCATGAAAGCCCCG – 3’ for CMV9-sLIGHT; and 5’ – ATGGAGCCTCCTGGAGACTG – 3’ and 5’ – TCAGTGGTTTGGGCTCCTC – 3’ for TNFRSF14.

Chromatin Immunoprecipitation (ChIP). The procedure for this assay is essentially described in Section 2.1.2 Materials and Methods. The purified DNA was then analyzed by PCR within linear amplification range followed by agarose gel electrophoresis. The following PCR primers were used: 5’ – CTGAGAGAGTGGGGTTGGAG – 3’ and 5’ – TGGGTCTTGCAAGGAAGAAG – 3’ for TNFRSF14 BS #1; 5’ – GCTGAGTTCCTCTGCTGGAG – 3’ and 5’ – CATGGGGAAGAGATCTGTGG – 3’ for TNFRSF14 BS #2.

Luciferase Activity Assay. TNFRSF14 promoter-containing fragment was amplified from human genomic DNA of H1299 cells and cloned into the pBVLuc luciferase reporter

vector containing a minimal promoter using the following primers: forward primer 5' – GCCTGGTGGGTCTATGACTG – 3' and reverse primer 5' – GTGGACGGAGTGGTGAGTG – 3'. Transfection of H1299 cells were performed in 24-well plate using 0.2 μg TIGAR luciferase reporter constructs, 0.05 μg p^{RL}-tk Renilla construct, and various amount of vectors expressing wild-type or mutant p53. Luciferase activities were measured 24 hours post-transfection using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized with Renilla luciferase activities to obtain the relative luciferase activity.

RNA Extraction and RT-qPCR. Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse Transcriptase kit (NEB). PCR analysis was carried out using Applied Biosystems 7500 Fast System with the following primers: 5' – GTGTCTGCAGTGCCAAATGT – 3' and 5' – CCACACACGGCGTTCTCT – 3' for TNFRSF14; 5' – AGCGAAGGTCTCACGAGGT – 3' and 5' – CGGTCAAGCTGGAGTTGG – 3' for LIGHT.

2.3.3. Results and Discussion:

TNFRSF14 is a novel p53 target

RNA-seq expression profile of inducible 3KR-expressing cells revealed that TNFRSF14 gene expression is upregulated upon p53 induction. To validate that TNFRSF14 gene is indeed a p53 target, we first examined the TNFRSF14 gene promoter for p53-binding sites. We found two potential binding sites (BS), with one site located ~1.7 kb upstream of the translational start site (TSS), while the other overlaps the TSS (**Figure 2.19A**). Next, we assessed whether p53 can bind to these potential binding sites by performing chromatin-immunoprecipitation (ChIP) assay in H1299 cells ectopically expressing human and mouse p53. PCR amplification of the immunoprecipitated DNA using primers that flank the binding sites showed enrichment of p53 binding at both BS #1 and #2, as well as the TIGAR promoter (**Figure 2.19B**).

Next, to evaluate whether p53 can transactivate the TNFRSF14 promoter, we generated a luciferase reporter construct containing a 910 bp fragment that overlaps BS #2 (**Figure 2.19A**). Co-transfection of Saos2 p53-null cells with TNFRSF14 reporter construct and mouse p53 resulted in an increase in luciferase activity compared to empty vector control (**Figure 2.20A**). Moreover, TNFRSF14 gene expression was activated in

Figure 2.19

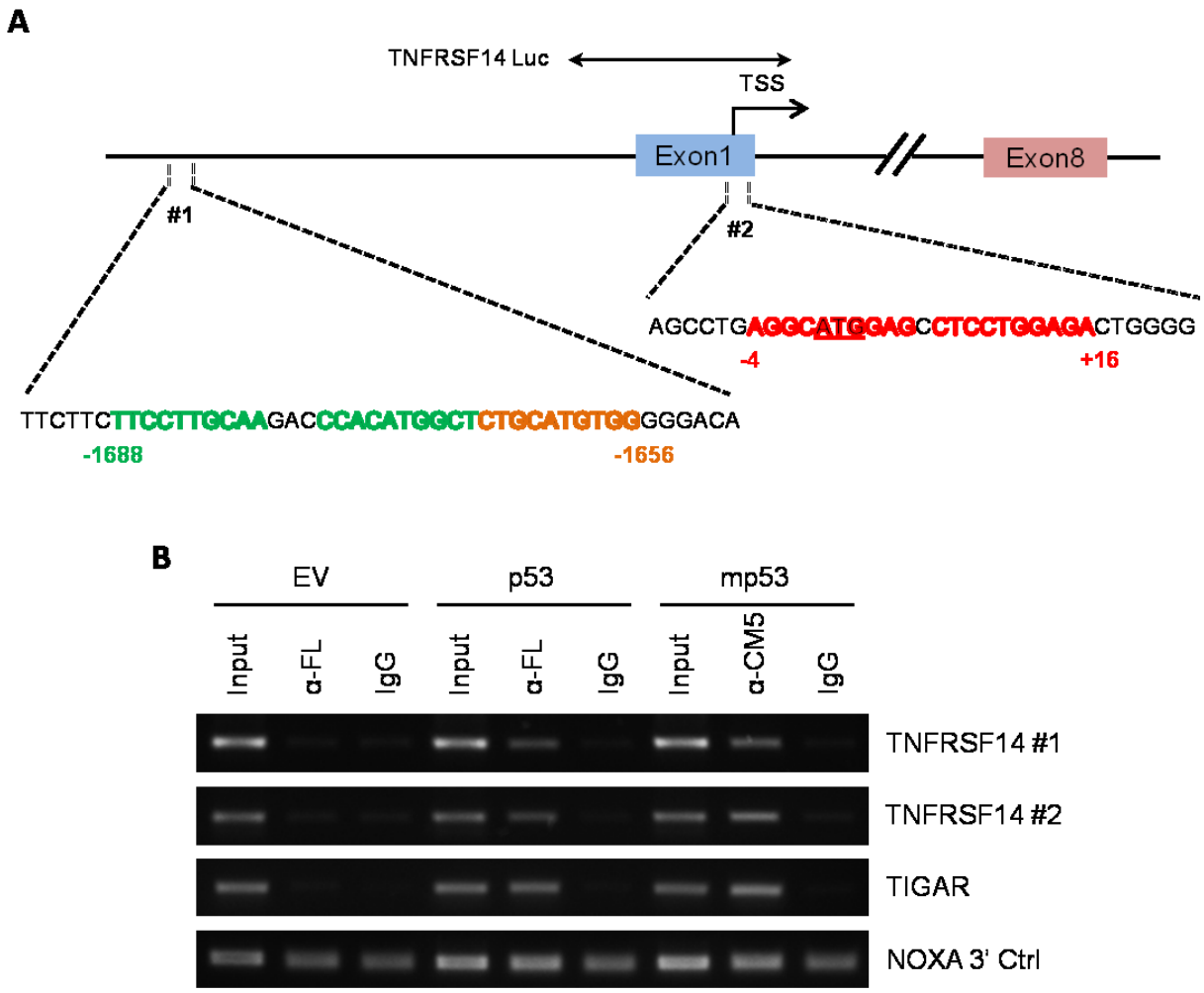

Figure 2.19. TNFRSF14 is a novel p53 target. (A) Cartoon figure depicts the promoter region of the TNFRSF14 gene that contains a p53 consensus binding site (BS) ~1.7 kb upstream of the translational start site (TSS) and another overlapping the TSS. A luciferase construct was generated by cloning the 910 bp fragment (TNFRSF14 Luc), which contains the #2 BS, upstream of a luciferase reporter gene. **(B)** Chromatin immunoprecipitation (ChIP) was performed using H1299 cells transfected with empty vector or vectors expressing human or mouse p53 for 24 hours. Short DNA fragments spanning the #1 and #2 BS of the TNFRSF14 locus, as well as the BS for the TIGAR gene, were amplified by PCR. The 3' non-binding region of the NOXA gene served as non-specific control.

Figure 2.20

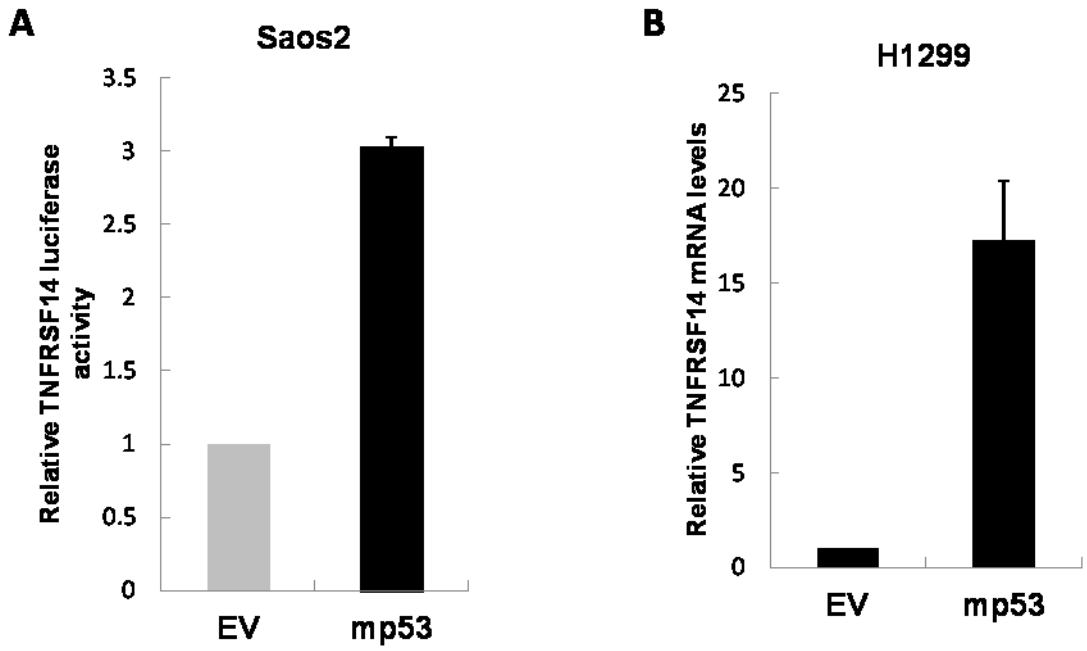

Figure 2.20. Transcription of TNFRSF14 is activated in the presence of p53. (A) TNFRSF14 luciferase construct (described in Figure 2.18A) was co-transfected with either empty vector or p53-expressing vector in Saos2 cells, and cell lysates were harvested 24 hours later and assayed for luciferase activity. **(B)** H1299 cells were transfected with either empty vector or p53-expressing vector, and total RNA were extracted 24 hours post-transfection. RT-qPCR was performed to determine the mRNA expression of the TNFRSF14 gene.

the presence of p53, as seen by the elevation of TNFRSF14 mRNA level in H1299 cells after transfection of p53 (**Figure 2.20B**).

To verify that the transactivation of the TNFRSF14 gene is indeed p53-dependent, we compared the induction of TNFRSF14 in the presence or absence of p53. Upon DNA damage treatment, HCT116 p53+/+ cells display increased TNFRSF14 expression over time, while the absence of p53 in HCT116 p53-/- led to no induction of the gene (**Figure 2.21A**). Similarly, TNFRSF14 expression was activated in MCF7 cells after DNA damage-induced p53 stabilization. However, MCF7 cells depleted of p53 failed to induce TNFRSF14 expression after the same drug treatment (**Figure 2.21B**). All together, these results demonstrate that TNFRSF14 is indeed a bona fide p53 target.

TNFRSF14 regulation is retained by 3KR p53, but not by 4KR98

Since p53-mediated upregulation of TNFRSF14 was identified in an expression profile of 3KR-expressing inducible cell line, we expect to verify this regulation in an overexpression system. Indeed, 3KR p53 was able to enhance TNFRSF14 gene expression, albeit at a lower level than wild-type p53. However, with an additional acetylation-ablating mutation at K98, the 4KR98 p53 lost transcriptional regulation on TNFRSF14, while the K98R p53 mutant showed no defect (**Figure 2.22A**). Similarly,

Figure 2.21

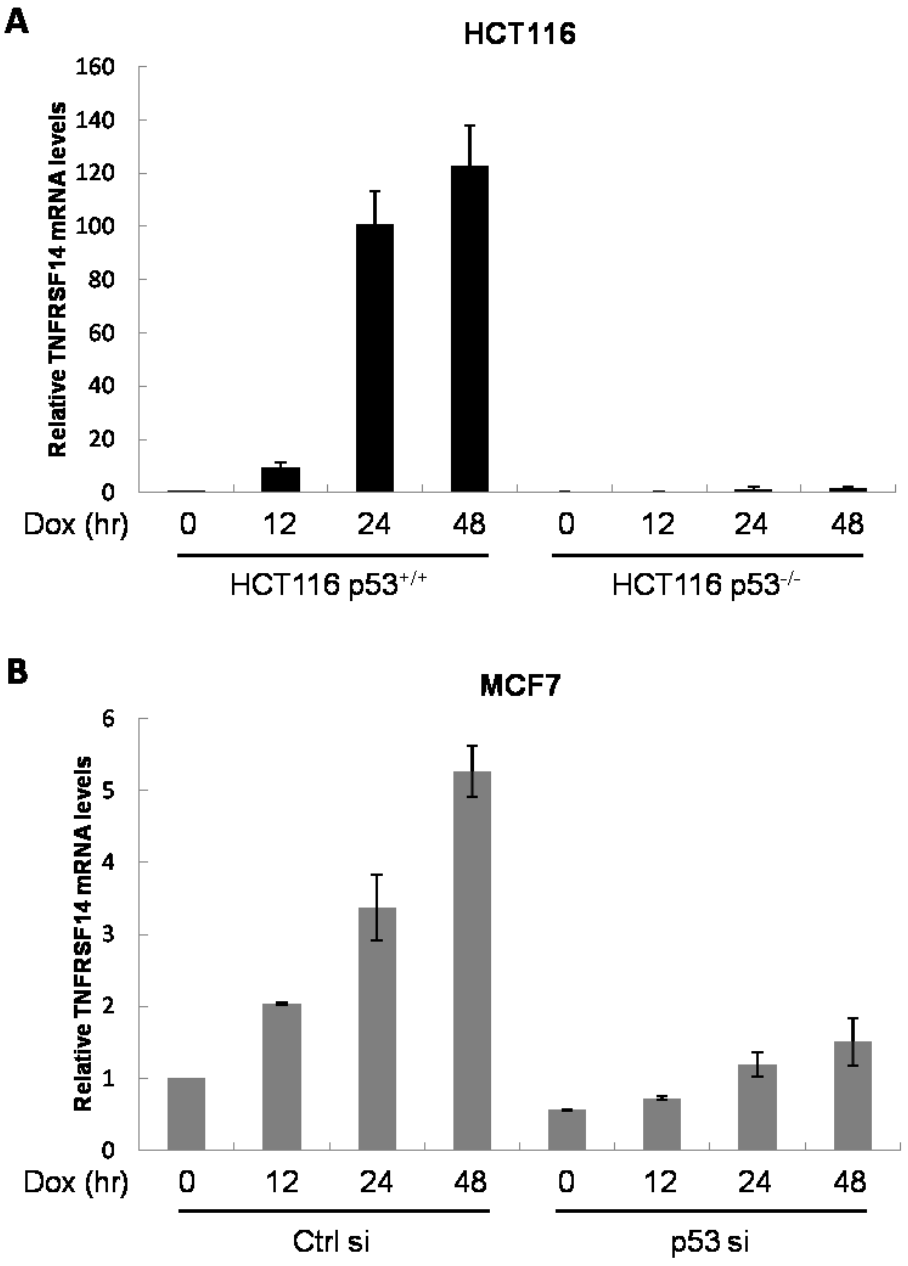

Figure 2.21. TNFRSF14 gene expression is p53-dependent. (A) HCT116 p53+/+ and p53-/- cells were treated with 0.2 μg/mL Doxorubicin for the times indicated and total RNA were extracted. RT-qPCR was performed to obtain the expression levels of TNFRSF14. **(B)** MCF7 cells were transfected with either non-specific or p53 siRNA, and were treated with 0.2 μg/mL Doxorubicin for the times indicated at 40 hours post-transfection. Total RNA were extracted and RT-qPCR was performed to determine TNFRSF14 expression.

Figure 2.22

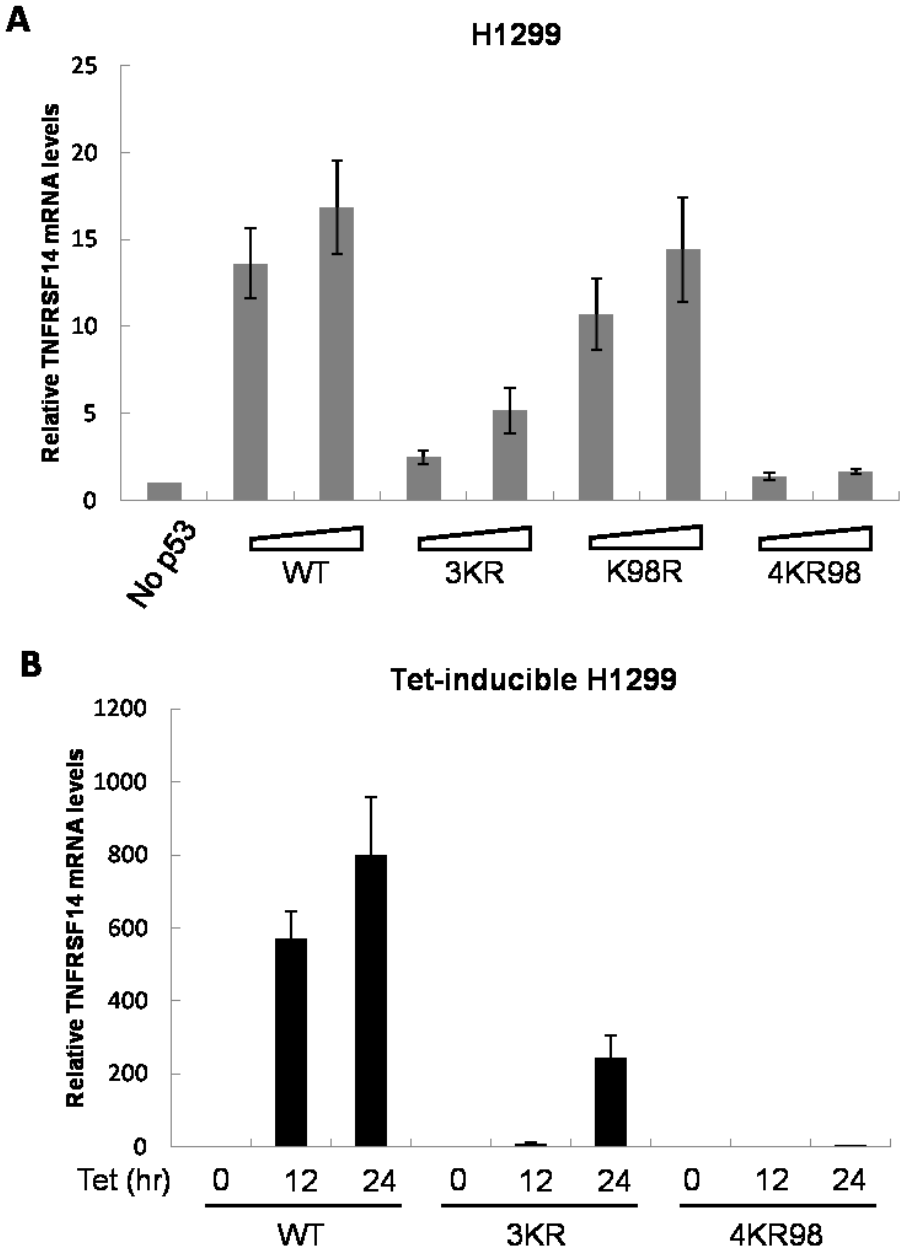

Figure 2.22. TNFRSF14 expression is abrogated by simultaneous mutation of 3KR and K98R. (A) H1299 cells were transfected with constructs expressing wild-type p53 and various p53 mutants as indicated for 24 hours, and total RNA were extracted for quantification of TNFRSF14 mRNA expression via RT-qPCR. **(B)** Tet-inducible H1299 cells that conditionally express wild-type, 3KR or 4KR98 p53 were induced with doxycycline for the times indicated, and total RNA were extracted to perform RT-qPCR for TNFRSF14 expression.

doxycycline induction of p53 expression in the Tet-on inducible cell lines resulted in upregulation of TNFRSF14 expression by wild-type and 3KR p53, but not by 4KR98 p53 (**Figure 2.22B**). To further support these findings, luciferase assay was performed by co-transfecting TNFRSF14 reporter construct with wild-type and various mutant p53 in Saos2 cells. While wild-type, 3KR and K98R p53 were all able to induce luciferase reporter activity, 4KR98 p53 exhibit significant defect in doing so (**Figure 2.23**). Interestingly, similar to previous observation of the DNA binding capabilities of p53 mutants (see Figure 2.18), 4KR98 p53 still retains the ability to bind TNFRSF14 promoter, as determined by ChIP assay (**Figure 2.24**). These findings show that 3KR p53, but not 4KR98, can activate TNFRSF14 gene expression, suggesting that the acetylation at K98 is critical for p53-mediated TNFRSF14 induction in the absence of K117/161/162 acetylations.

Stimulation of TNFRSF14 by LIGHT ligand leads to cell death

Previous studies have shown that activation of TNFRSF14 receptor by LIGHT ligand can trigger cell death in many different cell types. While the exact mechanism of how TNFRSF14 downstream signaling leads to cell death is unknown, evidence suggests that it is likely through an atypical pathway due to the long latency observed (2 - 5 days)

Figure 2.23

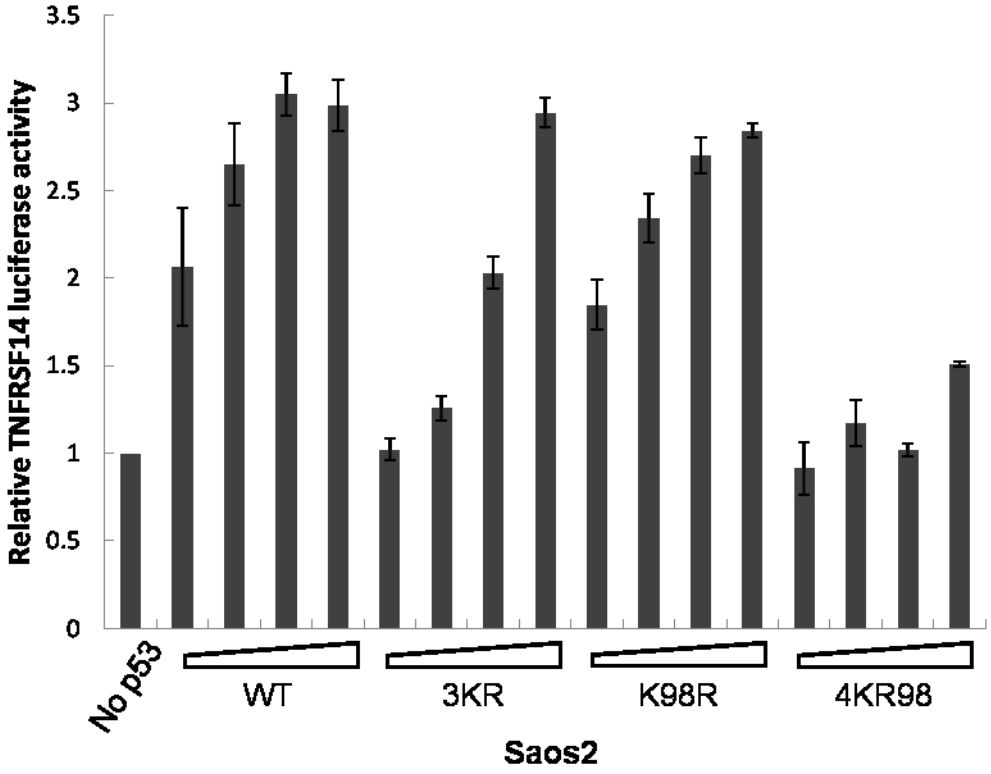

Figure 2.23. Defect in transactivating TNFRSF14 by 4KR98 is transcriptional in nature. Saos2 cells were co-transfected with TNFRSF14 luciferase construct (described in Figure 2.18A) and increasing amount of vectors expressing either wild-type, 3KR, K98R or 4KR98 p53. At 24 hours after transfection, cell lysates were collected and assayed for luciferase activity.

Figure 2.24

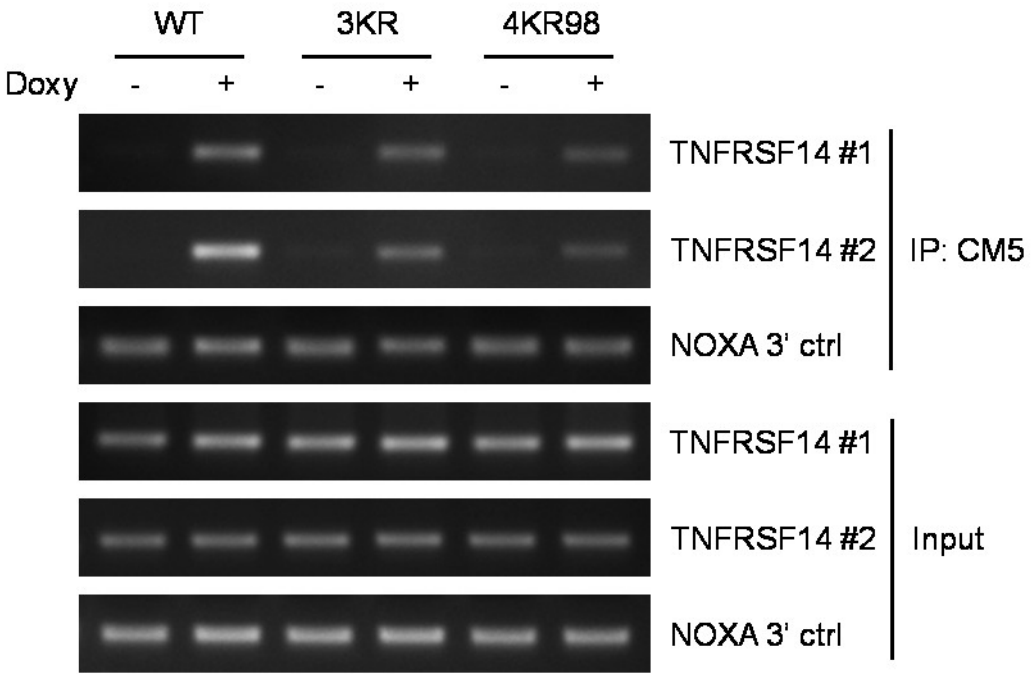

Figure 2.24. Ablation of K98 acetylation does not affect DNA binding to TNFRSF14 promoter. Tet-inducible H1299 stable lines that conditionally express wild-type, 3KR or 4KR98 p53 were either untreated or induced with doxycycline (5 μg/mL) for 8 hours. Cells were then crosslinked with formaldehyde, and DNA-bound p53 were immunoprecipitated for ChIP assay.

before cell death occurs [130,131]. To confirm the TNFRSF14-mediated cell death observed in previous studies, we ectopically expressed membranous and soluble LIGHT ligand in TNFRSF14-positive cells. Indeed, expression of LIGHT ligand in MCF7 breast and HT29 colon adenocarcinomas that express TNFRSF14 led to cell death 48 hours after transfection (**Figure 2.25**). Moreover, we generated inducible TNFRSF14-expressing cell line using H1299 lung adenocarcinoma that lacks, or maintain very low basal levels of, TNFRSF14 expression to further validate the dependency on TNFRSF14 in LIGHT-induced cell death. As expected, doxycycline-induction of TNFRSF14 expression in the H1299 stable line resulted in cell death in the presence of LIGHT ligand at 72 hours post-transfection, but not in the absence of LIGHT or TNFRSF14 expression (**Figure 2.26A** and **2.26B**).

In order to determine the type of cell death associated with TNFRSF14, we attempted to prevent cell death using inhibitors of various pathways. We transfected inducible TNFRSF14-expressing H1299 cells with LIGHT ligand in the presence of inhibitors for apoptosis (Z-VAD-FMK), autophagy (3-methyladenine), necroptosis (necrostatin-1), or ferroptosis (ferrostatin-1). Surprisingly, treatment of Z-VAD-FMK caspase inhibitor, but not others, was able to prevent cell death, suggesting that TNFRSF14-mediated death is likely apoptotic in nature that involves caspase cascade

Figure 2.25

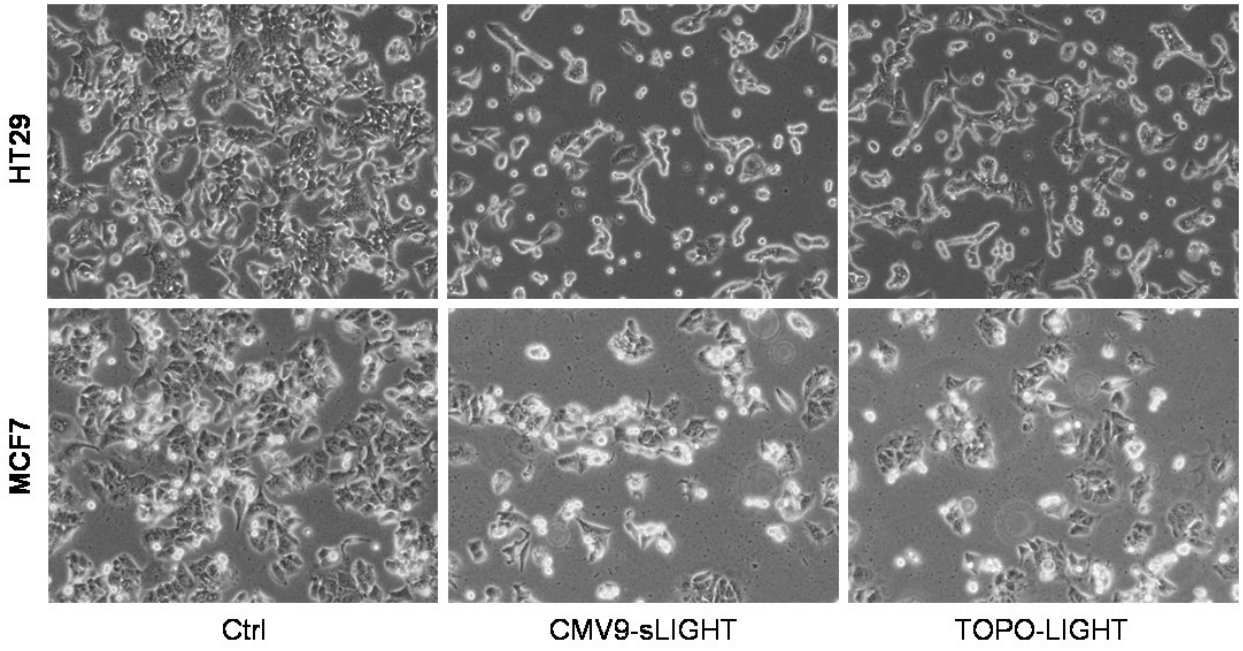

Figure 2.25. Stimulation of TNFRSF14-positive cells by LIGHT ligand leads to cell death. HT29 and MCF7 cells were transfected with either empty vector or vectors expressing secreted (CMV9-sLIGHT) or full-length (TOPO-LIGHT) LIGHT ligand. Cell death was observed at 48 hours after transfection.

Figure 2.26

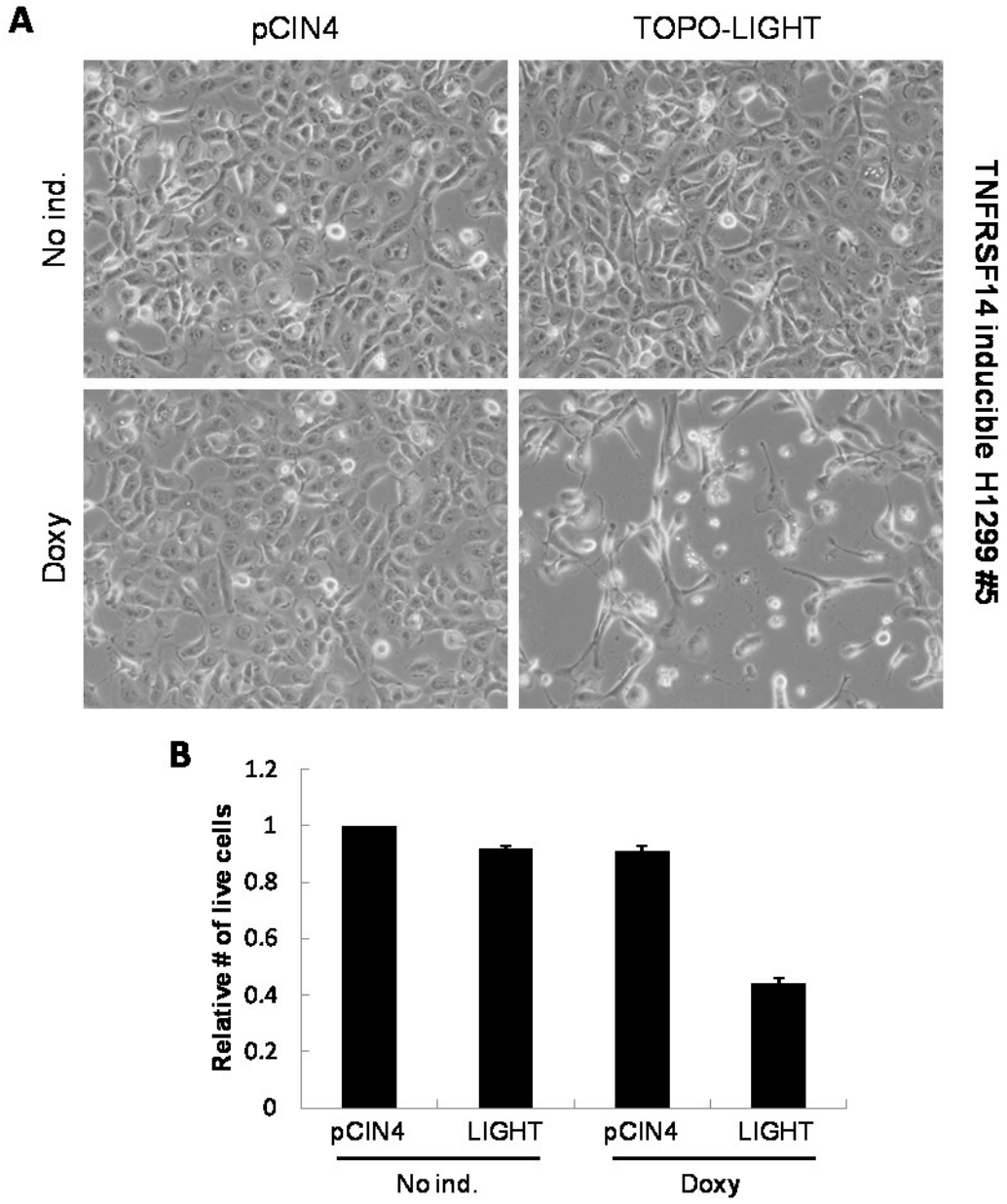

Figure 2.26. LIGHT-induced cell death is dependent on TNFRSF14. (A) Inducible H1299 cells conditionally expressing TNFRSF14 were transfected with either empty vector or vector expressing full-length LIGHT ligand, and were then either left untreated or treated with doxycycline (5 μg/mL) for 72 hours. **(B)** The relative number of live cells was counted for (A), and the graph depicts the average of three separate experiments.

Figure 2.27

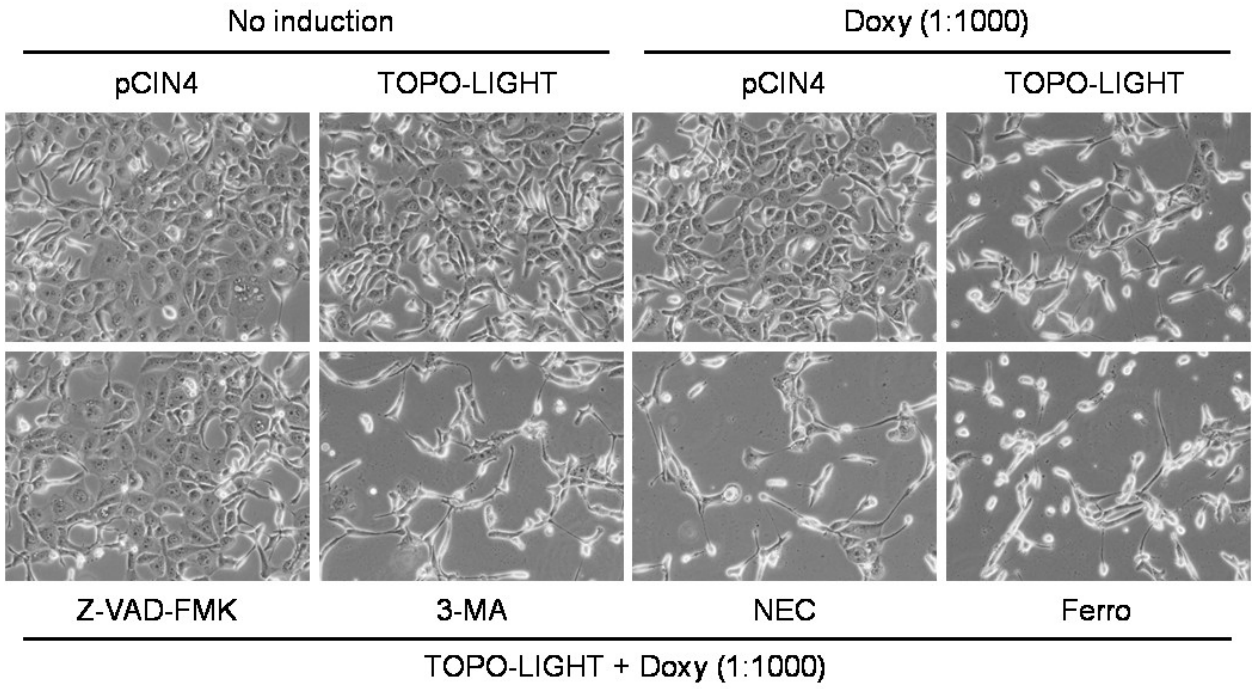

Figure 2.27. TNFRSF14-mediated cell death can be inhibited by caspase inhibitor. TNFRSF14 inducible H1299 cells were transfected with empty vector or vector expressing full-length LIGHT ligand. At 4 hours post-transfection, cells were either left untreated or treated with doxycycline (5 μg/mL) for 72 hours in the presence of Z-VAD-FMK (pan caspase inhibitor), 3-MA (3-methyladenine, autophagy inhibitor), NEC (necrostatin-1, RIP1 inhibitor for necroptosis), or Ferro (ferrostatin-1, ferroptosis inhibitor).

signaling (**Figure 2.27**). All together, these findings indicate that LIGHT ligand can trigger TNFRSF14-mediated caspase-dependent cell death with unusually long latency.

LIGHT ligand can trigger TNFRSF14-mediated cell death in 3KR, but not 4KR98, cells

Since 3KR p53 is defective in promoting apoptosis through the transactivation of pro-apoptotic genes, but retains the ability to regulate TNFRSF14, we wish to investigate whether induction of 3KR p53 can lead to LIGHT-dependent cell death. To test this, we utilized Tet-on inducible p53-expressing H1299 stable lines in conjunction with ectopically-expressed LIGHT ligand. As expected, in the absence of p53-mediated induction of TNFRSF14, cells expressing LIGHT did not undergo cell death. However, with the expression of 3KR p53, LIGHT-expressing cells underwent cell death after 72 hours of doxycycline-induction and transfection, similar to that observed in Tet-on inducible TNFRSF14-expressing H1299 (**Figure 2.28A**). Interestingly, cells expressing 4KR98 p53, which lack TNFRSF14 expression, did not undergo cell death upon LIGHT expression (**Figure 2.28A**). **Figure 2.28B** shows the corresponding expression of p53 and LIGHT ligand, while **Figure 2.28C** shows the quantification of live cells in this experiment. Together, our results underscore a novel non-canonical p53-regulated

Figure 2.28

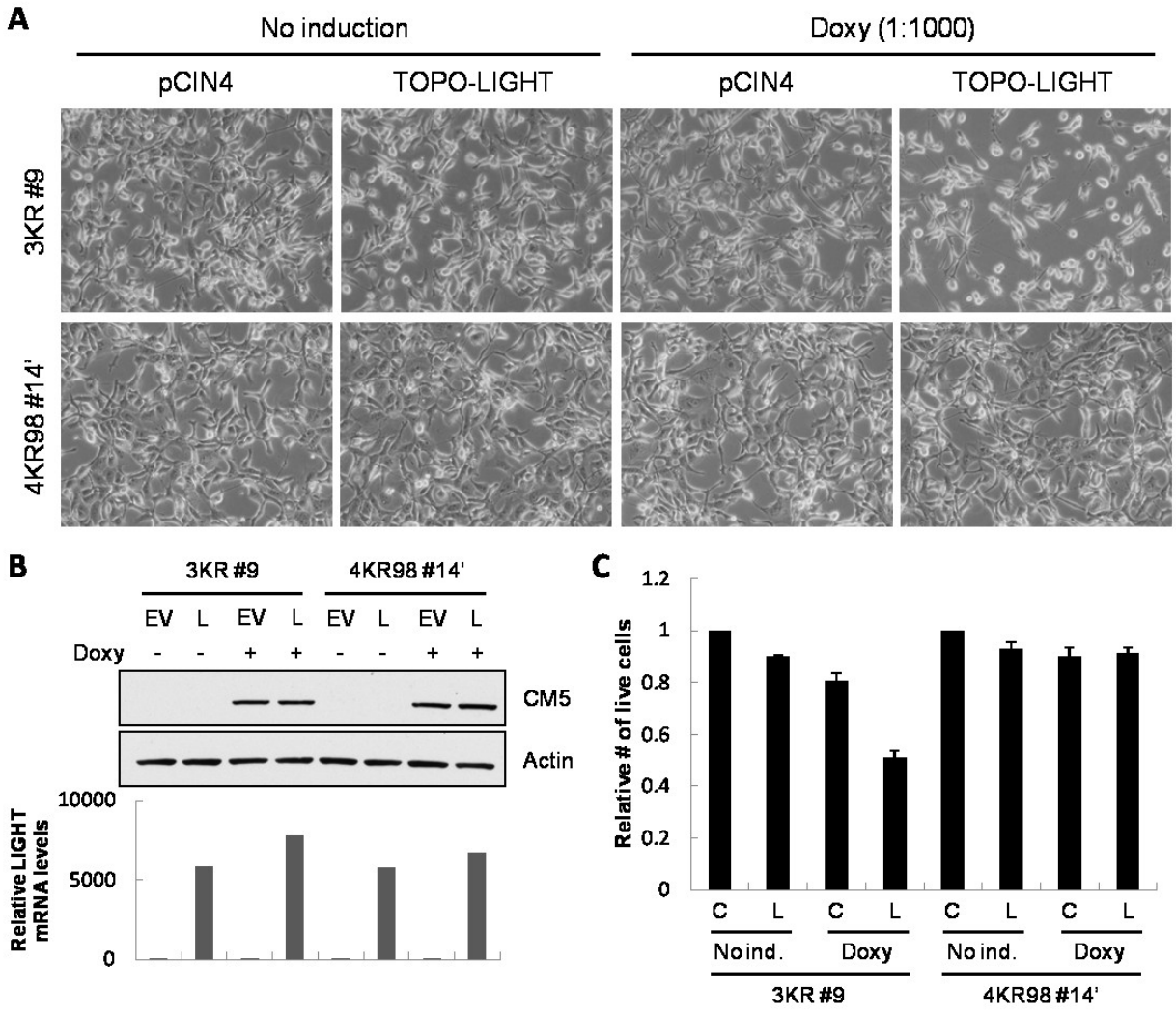

Figure 2.28. LIGHT expression triggers cell death after induction of 3KR p53, but not 4KR98. (A) Tet-on inducible H1299 conditionally expressing 3KR or 4KR98 p53 were transfected with either control vector or vector expressing full-length LIGHT ligand. Cells after 4 hours of transfection were left untreated or treated with doxycycline (5 μg/mL) for 72 hours to induce p53 expression and subsequent cell death. **(B)** Quantification of p53 and LIGHT expression via Western blotting and RT-qPCR, respectively. **(C)** Relative number of live cells was calculated from experiments shown in (A), and the graph represents the average of three separate experiments.

pathway through TNFRSF14 signaling that potentially plays a crucial role in tumor suppression.

3. Discussion and Future Directions

Our understanding of how p53 functions as a tumor suppressor has evolved tremendously since its discovery more than three decades ago. Apoptotic and growth arrest functions of p53 have been studied extensively, and have proven to play critical roles in maintaining genomic stability through repair or elimination. Later studies uncovered diverse array of p53-mediated cellular functions, including metabolism, autophagy, senescence, and aging, which contribute significantly to p53 tumor suppressive activity. More interestingly, p53 can even go beyond its tumor suppressive role and participate in cellular homeostasis and energy balance.

3.1. Identification of PanK1 as a novel p53 metabolic target

Recent findings have demonstrated the importance of p53 in metabolic regulation. In our first study, we have provided evidence that linked p53 function to CoA metabolism through a novel p53 metabolic target PanK1, an enzyme that catalyzes the rate-limiting step of CoA synthesis. PanK1 was first identified as a potential p53 target from a ChIP-on-chip analysis, and subsequent luciferase reporter mutagenesis and gel shift assay revealed two p53 binding sites on the 5'-end of *PANK1* exon 1α, indicating that PanK1 is likely a direct p53 target. Expressions of both α- and β-isoforms of PanK1 are shown to be induced by DNA damage in a p53-dependent manner, although the

presence of PanK1 expression in p53-null cells suggests concomitant regulation by p53-independent pathways as well.

Many of the previously identified p53 metabolic targets play significant roles in modulating the effect of p53-dependent apoptosis or growth arrest. GAMT, while not sufficient to trigger cell death, is required to induce the full apoptotic response after p53 activation [138]. Similarly, it has been shown that TIGAR may partially rescue cells from p53-induced apoptosis, while TIGAR mutants exhibit impaired anti-apoptotic activity [39]. To our surprise, although PanK1 expression is upregulated under p53 activation in response to genotoxic stress, depletion of PanK1 by siRNA targeting did not affect p53-mediated cell death after DNA damage. Our findings suggest that p53 regulation on PanK1 expression may instead play a role in metabolic settings.

PanK1 is most highly expressed in the liver, in which it has been shown to carry out proper metabolic transformations during fasting that are mediated by adequate CoA synthesis; high CoA abundance is required for fatty acid and amino acid degradations that generate ketone bodies and glucose as alternative fuel sources under nutrient deprivation (**Figure 3.1**) [125,127]. Indeed, PanK1 knockout mice exhibit reduced fatty acid oxidation and gluconeogenesis during starvation due to lower availability of free CoA, which led to accumulation of long-chain acyl-CoA and triglycerides in the liver and

Figure 3.1

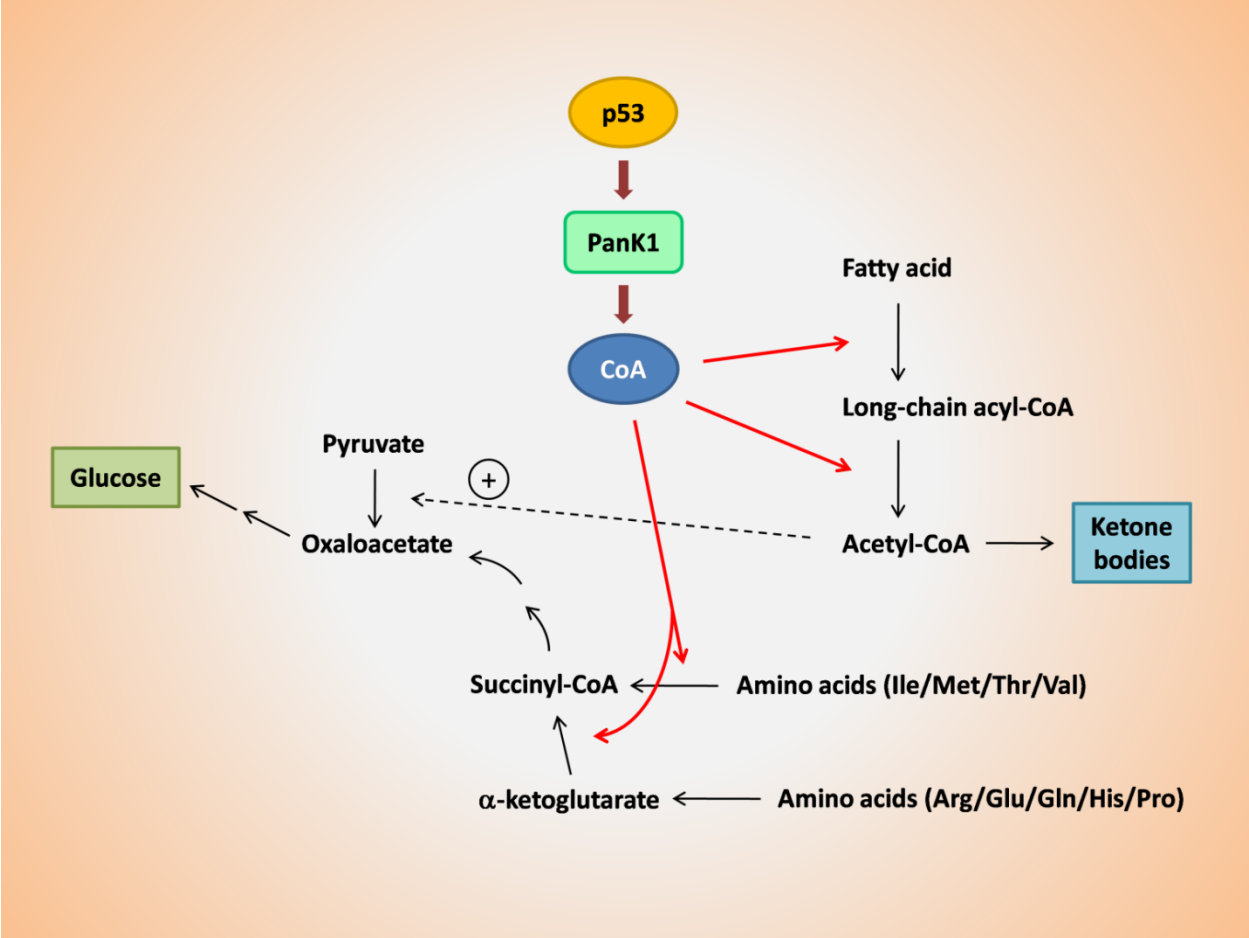

Figure 3.1. A model of p53-mediated PanK1 function through CoA synthesis. During starvation, p53 maintains PanK1 expression in the liver and provides adequate CoA synthesis to confer energy homeostasis. CoA is required for β-oxidation of fatty acids to generate acetyl-CoA, which is used as substrate for ketone production. CoA is also required for amino acid conversion to TCA-cycle intermediates, which can be shuttled into the cytoplasm from the mitochondria to undergo gluconeogenesis. Production of both ketone bodies and glucose in the liver during starvation is crucial for sustaining energy source for all other organs and tissues. Note that the conversion of pyruvate to oxaloacetate is subjected to positive allosteric regulation by acetyl-CoA, whose level is dependent on β-oxidation and CoA availability.

lowered blood glucose levels [127]. Interestingly, gene expressions of all PanK isoforms are not elevated during fasting, implying that the existence of other factors, such as allosteric regulation, may be important in governing CoA levels [126,127]. Nevertheless, in light of the p53 → *PANK1* axis, we hypothesized that metabolic stress such as starvation may at least lead to maintenance of *PANK1* gene expression through p53 activity. To our surprise, while PanK1 expression remained unchanged after glucose starvation in HCT116 $p53^{+/+}$ cells, its $p53^{-/-}$ isogenic counterpart exhibited significant decrease in PanK1 protein levels, suggesting that p53 is required for the maintenance of *PANK1* gene expression under energy deprivation. Consistent with this finding, we observed a substantial reduction in the total liver PanK activity as well as the liver free CoA levels in fasting p53-null mice. Moreover, the p53-null mice is reminiscent of the PanK1-deficient mice in that there are significantly greater accumulation of fatty acid and triglyceride after fasting, along with lower fasting blood glucose compared to WT mice, demonstrating impairment in both CoA-dependent fatty acid oxidation and gluconeogenesis.

One interesting discovery in our study is that PanK1 expression appears to be p53-independent under conditions of excess nutrients. The transition from p53-independent to p53-dependent regulation of PanK1 after starvation implies that the

unavailability of specific nutrients triggered a switch in the transcriptional machinery responsible for PanK1 expression. Previous works have shown that the activity of Sp1, a ubiquitously-expressed housekeeping transcription factor that regulates the expression of thousands of genes implicated in a diverse array of cellular processes, is dependent upon the cellular nutritional and energy status. Fasting, glucose starvation and hypoxia can cause degradation of Sp1 or downregulation of the DNA binding activity of Sp1 via post-translational modifications such as O-linked glycosylation at serine/threonine residues [139-141]. One possibility is that PanK1 expression is regulated by Sp1 during normal physiological conditions. However, in the face of energy deprivation, Sp1 activity diminishes to conserve nutrients, while p53 is activated in response to metabolic stress to maintain the expression of PanK1. Testing this hypothesis would allow us to gain better insight on how the metabolic response of p53 fits in the complex dynamics of energy homeostasis.

In this study, we performed ChIP-on-chip assay using an *in vitro* approach with H1299 p53-null cells ectopically expressing human p53. The rationale behind this screening method in an artificial system is to be able to identify previously unknown metabolic targets of p53. As a result, we were able to establish the p53 → PanK1 axis and linked p53 function to CoA-dependent metabolism in the liver during starvation. A

more specific approach, given that we now know the cell-type localization and specific trigger of this p53 function, would be to perform the p53 ChIP assay using liver cells after starvation. First, we would expect to see enrichment of p53 binding on the *PANK1* promoter in the liver cells of mice subjected to starvation compared to those that were fed. Second, performing a p53 ChIP-on-chip using this *in vivo* system would potentially elucidate other downstream mediators of p53 that may also contribute to p53-dependent regulation of energy homeostasis.

3.2. Novel human K101/mouse K98 p53 acetylation contribute to differential regulation

Our identification of a novel p53 metabolic target ultimately led to an increasingly puzzling question of how p53 can differentially regulate a vast number of functionally diverse target genes in a context-specific manner. This question brought about our second study, which sought to uncover novel p53 post-translational modifications that may contribute to differential regulation of downstream p53 targets. From previous studies in our lab, we have shown that post-translational modifications of p53, particularly acetylation of lysine residues, play a crucial role in promoter-specific transcriptional activation of p53 targets. The K120 acetylation of p53 is required for

transactivation of proapoptotic Puma gene, while K164 and C-terminal acetylations contribute to the activation of p21 growth arrest gene, but none is necessary for the induction of Mdm2 expression [93,95]. Similar observations were made in the 3KR mouse model, in which both apoptotic and growth arrest functions were ablated, while regulation on Mdm2 and metabolic targets remained intact [107]. Based on these findings, we screened for novel p53 modifications using mass spectrometry and identified a new acetylation site at lysine K101 on human p53 (K98 on mouse p53). The acetylation, catalyzed by CBP, was confirmed using in-house site-specific antibody generated against Ac-K101 p53. Acetylation at K101 in human p53 and K98 in mouse p53 lie within the DNA-binding domain, and has been shown to be mutated in human cancer.

We observed that ablation of acetylation at K98 in mouse p53 alone did not affect transcriptional activity of p53. Interestingly, when simultaneously disrupting acetylations at K117/161/162 and K98, transactivation of targets such as Mdm2 and Tigar, which are regulated by 3KR p53, was compromised. Collectively with previous findings, our data suggest that the acetylations in the DNA-binding domain of p53 may have redundant roles in promoter-specific p53 activation. Activation of pro-apoptotic targets such as Puma and Bax require K117 acetylation. On the other hand, p21 growth arrest induction

can be achieved by mouse p53 with either K117 or K161/162 acetylation; only ablation of acetylation at all three sites will p53-mediated p21 expression be abolished. Moreover, activation of targets such as Mdm2 and Tigar by mouse p53 requires only either K98 acetylation or acetylation(s) at K117/161/162. While which of the three acetylations at K117/161/162 could compensate for K98 acetylation in activating Mdm2/Tigar has not been tested, it is clear from our data that K98 acetylation is required in the absence of K117/161/162 acetylations. These observations indicate that different p53 acetylation or combinations of acetylations can be sufficient to activate certain p53 targets. In other words, many p53 acetylation sites can be redundant for activating a given target, and that the absence of one acetylation can be compensated by another to achieve gene expression.

In light of these findings, we propose an expansion of our working hypothetical model detailed in Section 1.5 (**Figure 3.2**). In the absence of cellular stress, K98 acetylation in mouse p53 may constitute as minimal modification required for p53-mediated expression of genes such as Mdm2 and Tigar. Functionally, expression of Mdm2 and Tigar under non-stressed condition is appropriate, as Mdm2 keeps p53 levels under tight control, while Tigar activity may contribute to normal cellular energy homeostasis. Since K98 acetylation is not sufficient to induce growth arrest and

Figure 3.2

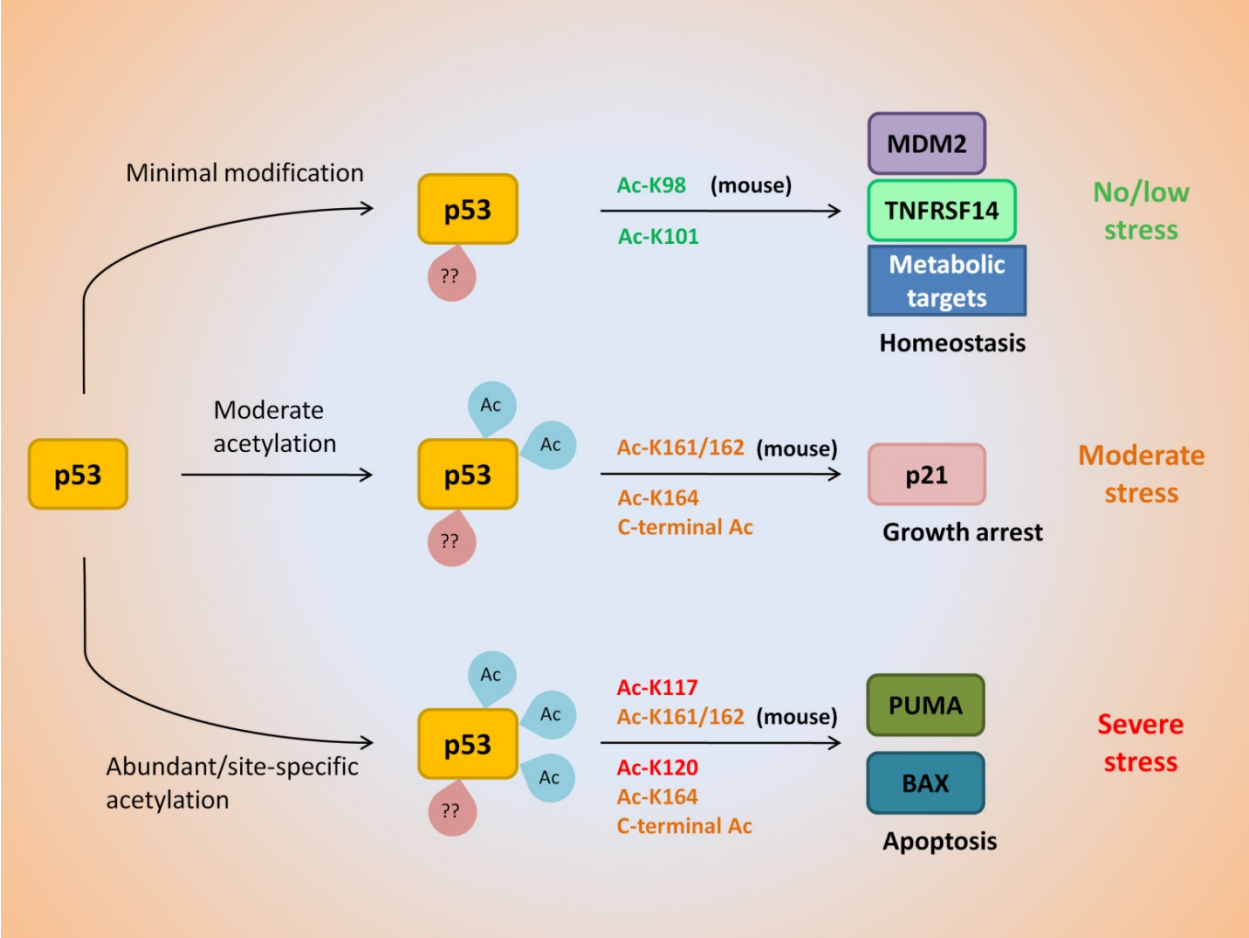

Figure 3.2. An expanded model of differential regulation by p53. Identification of human K101/mouse K98 acetylation site further contributed to our working model. The human K101/mouse K98 acetylation may constitute the minimal modification for p53 basal activity.

apoptotic targets, it is a harmless form of p53 activation that can occur in the absence of stress. As cells encounter stress, acetylation of p53 increases depending on the stress level, with mild stress triggering growth arrest through K161/162 acetylations, while severe stress causing apoptosis via K117 acetylation.

Even though the acetylation sites in the DNA-binding domain play crucial roles in p53 activation and differential regulation, our data suggest that they do not appear to significantly affect promoter-specific binding. For example, we observed that wild-type, 3KR, and 4KR98 p53 can all bind p21 and TIGAR promoters, yet these varieties of p53 possess drastically different transcriptional activity on p21 and TIGAR gene. One possibility is that the acetylations in the DNA-binding domain alters the conformation of the p53, and in turn, influences co-activator or transcriptional machinery recruitment instead of affecting DNA binding. Co-activators, usually histone acetyltransferases (such as CBP/p300, PCAF, GCN5 and Tip60), are recruited by transcription factors to acetylate histones and relieve the coiling of chromatin to allow transcription to occur. One study showed that acetylation at lysine residues K320/373/381/382 were involved in recruitment of co-activators such as CBP and TRRAP that led to histone acetylation of the p21 promoter [142]. Furthermore, evidence indicate that DNA binding domain and the C terminus of p53 may contain docking sites for several important p53 co-activators

such as ASPPs, 53BP1, Tip60/hMOF, hCAS/CSE1L, and HZF, which are all critically involved in the induction of different p53 targets [92,93,143-147]. Thus, it is possible that different acetylations or combinations of acetylations may present as a form of “code” for association with various co-activators, and therefore, allow different p53 target to be expressed depending on the co-activator present. Testing whether different co-activators are recruited based on the presence of p53 modifications in a promoter-specific manner could shed light on the mechanism behind differential regulation by p53.

3.3. TNFRSF14 is a novel non-canonical p53 target that may contribute to p53-mediated tumor suppression

Aside from understanding how p53 can differentially regulate its downstream targets, our study on the novel acetylation at K101 also hope to elucidate the mechanism underlying p53-mediated tumor suppression. The 3KR mouse model, as well as evidence from other studies, demonstrates that apoptotic and growth arrest functions of p53 may not be essential for suppressing tumor formation (see Section 1.4). Furthermore, the preservation of p53-mediated metabolic function in the 3KR mice initially prompted interest in linking cellular metabolism to tumor suppression, but later studies revealed that the opposing roles of p53 metabolic genes in both tumor

suppression and tumor survival may be difficult to reconcile. Therefore, we decided to identify novel mediators of p53 tumor suppressor function in hope of better understanding of how p53 prevents cancer. Since 3KR p53 retains the ability to suppress spontaneous tumor growth, we profiled gene expression in H1299 cells stably transfected with Tet-inducible 3KR p53 expression vector and identified a novel p53 target, TNFRSF14.

TNFRSF14, as described in Section 2.3.1, is a transmembrane receptor in the TNF receptor family of proteins. The reason for our interest in this particular target was two-fold. First, stimulation of the TNFRSF14 receptor can lead to cell death in various cancer cells [130-133]. Although the exact mechanism of how TNFRSF14 leads to cell death is unclear, it may represent a unique type of tumor suppression that can potentially link to immune surveillance (discussed below). Second, TNFRSF14 gene is recently found to be highly mutated in follicular lymphomas and diffuse large B-cell lymphomas, and may potentially be found to be mutated in other cancers as well if such studies were performed [134-137]. Notably, TNFRSF14 is the first p53 target identified that is mutated in cancer; even canonical p53 targets such as p21, Puma and Bax are not mutated in human cancer in any noticeable frequency.

Our results show that TNFRSF14 is indeed a bona fide p53 target. Interestingly,

regulation on TNFRSF14 expression is retained by 3KR mouse p53, but not by 4KR98 mutant, which is similar to the expression kinetics observed for Tigar and Mdm2. Parallel to what we have observed for other p53 targets, mouse p53 binding on TNFRSF14 promoter was not significantly affected by the K98 acetylation status, suggesting that other mechanism for altering p53 promoter-specific transcriptional activity may be at play (as discussed earlier).

The TNFRSF14 receptor recognizes and binds to LIGHT ligand, which is also a transmembrane protein. Stimulation of the TNFRSF14 receptors by LIGHT ligand causes cell death in various cancer cell lines, both in our hands and in other published works. The TNFRSF14-mediated cell death can be inhibited by pan caspase inhibitor (Z-VAD-FMK), suggesting that the apoptotic pathway is likely involved. However, the long latency observed before cell death occurs is unusual for apoptotic responses, such as ones triggered by DNA damage or hypoxia. TNFRSF14 receptor, unlike many other TNF receptors, do not contain cytoplasmic death domain, and therefore, cannot directly induce cell death [148]. However, TNFRSF14 activates downstream NF-κB pathway during T-cell activation, and therefore, it is possible that TNFRSF14-mediated apoptosis is carried out by the same pathway. NF-κB pathway typically promotes cell proliferation and induces anti-apoptotic effects, and aberrant hyperactivity of NF-κB is often found in

cancer [149,150]. Surprisingly, recent evidence also hints on the possibility of pro-apoptotic function mediated by NF-κB through the transcription of Fas receptor [151]. Coincidentally, a study showed that stimulation of the TNFRSF14 receptor in non-Hodgkin's lymphoma sensitized cancer cells to Fas-induced apoptosis [133]. This convoluted hypothetical axis of TNFRSF14 → NF-κB → Fas could partially explain the long latency prior to the initiation of cell death. Other possibilities also exist through TNFRSF14-associated TRAF proteins, although many of the downstream pathways predominantly promote cell survival (JNK/AP-1/ERK). The TNFRSF14-mediated cell death pathway can be further characterized by testing the aforementioned possibilities, or through identification of mediators of downstream signal cascade via complex purification.

As mentioned previously, 3KR mice are not prone to spontaneous tumor formation in the absence of canonical apoptotic and growth arrest functions. Our findings that link p53 to TNFRSF14 function suggest that TNFRSF14 may potentially contribute to 3KR p53-mediated tumor suppression. Under *in vitro* conditions, we observed that TNFRSF14 expression induced by 3KR p53 triggered cell death in the presence of LIGHT ligand. How this novel mechanism translates into tumor suppression *in vivo* is of great interest for our lab. Based on what is currently known for TNFRSF14 receptor, it is

likely that tumor suppression *in vivo* may involve cross-talks and interactions between host cells and the immune system. It would be critical to generate the 4KR98 knock-in mice with additional mutation at K98 to determine if there is a loss of tumor suppressive function compared to the 3KR mice. It would also be interesting to generate TNFRSF14 knockout in the 3KR mice to examine whether the TNFRSF14 gene specifically mediates tumor suppression in the 3KR p53 background.

TNFRSF14 receptor is widely expressed in different cell types, including lymphocytes and hematopoietic cells (monocytes, dendritic cells, neutrophils, and natural-killer cells), as well as parenchymal cells [148]. LIGHT ligand, on the other hand, is much more tightly regulated, and its expression is limited to lymphocytes, monocytes and immature dendritic cells [152]. In T and B lymphocytes, TNFRSF14 expression is high on resting T cells and on naïve and memory B cells, but is downregulated on activated T and B cells [153,154]. Interestingly, the expression of LIGHT ligand on lymphocytes is reciprocal to that of TNFRSF14. Upon T and B cell activation, LIGHT ligand expression increases, while TNFRSF14 expression falls [155]. This expression dynamics between the receptor and ligand is presumably to prevent induction of TNFRSF14-mediated cell death among the cell population. Moreover, expression of LIGHT ligand on activated T and B cells and dendritic cells would hypothetically allow for

tumor immune surveillance to take place.

Further studies are clearly required to elucidate the role in which TNFRSF14 plays to confer tumor suppression. Nonetheless, we propose a theoretical model here to serve as a guide for future directions (**Figure 3.3**). Tumor immune surveillance is a critical step in checking tumor cell growth in the event that intrinsic tumor suppression mechanisms fail [156]. Oftentimes, transformed cells accumulate changes that causes intrinsic resistance to apoptosis, ie. overexpression of Bcl-2 or Bcl-X_L. In such cases, tumor cells will expose tumor-specific antigens on the cell surface via antigen-presenting major histocompatibility complex (MHC), which will be recognized by CD8+ killer T cells [157,158]. In this scenario, p53-mediated TNFRSF14 expression could serve as a co-existing pathway for immune-targeted tumor cell elimination, or even function as a failsafe mechanism in the event that tumor cells escape immune surveillance. As mentioned earlier, activated lymphocytes express LIGHT ligand on the cell surface, and during lymphocyte infiltration into the tumor bulk, these lymphocyte can trigger TNFRSF14-mediated cell death through LIGHT ligand binding (in addition to MHC recognition and elimination). Moreover, as the process of immune surveillance progresses, tumor cells evolve to escape immune detection by disrupting antigen processing pathway or downregulation of the MHC antigen-presenting molecules

Figure 3.3

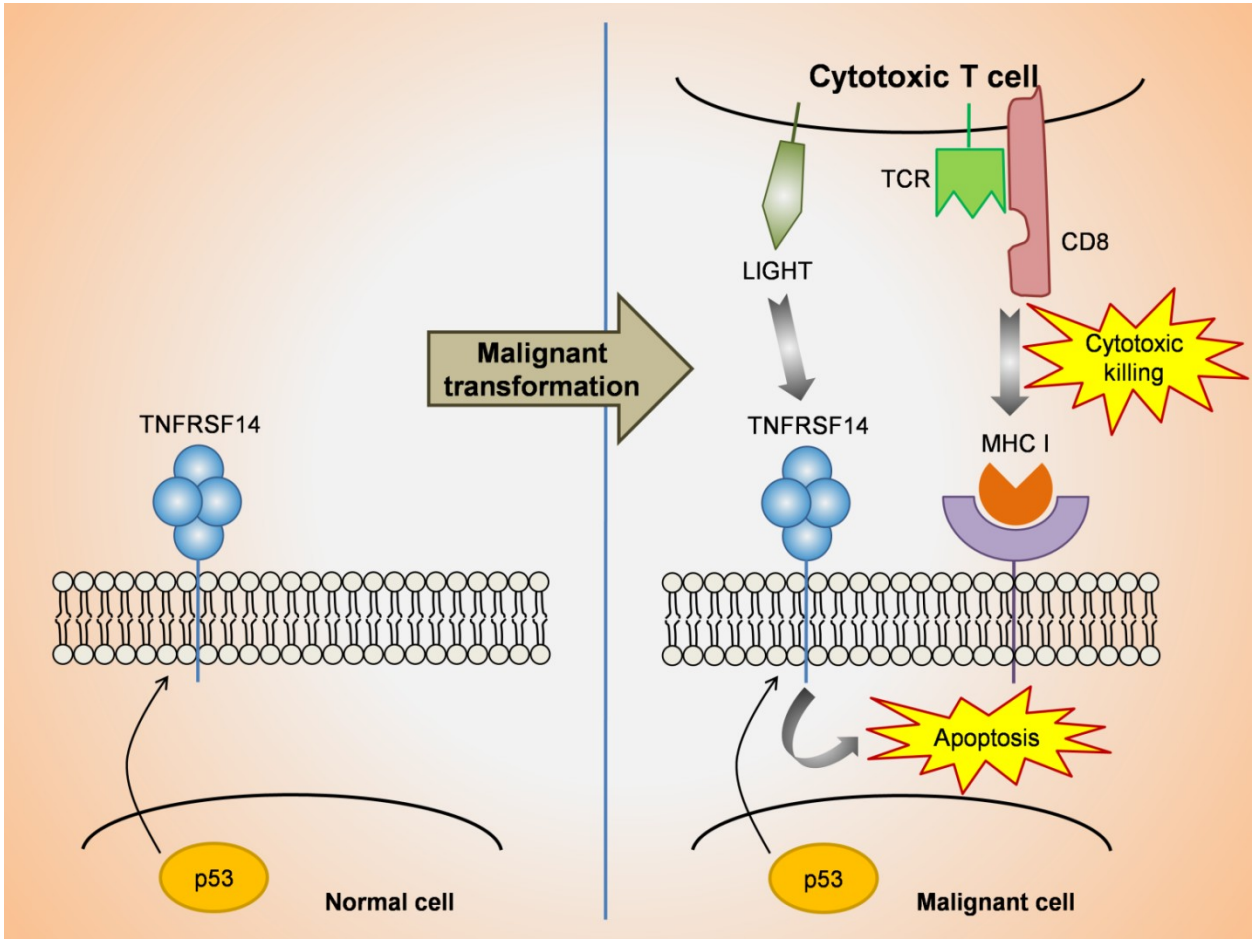

Figure 3.3. A hypothetical model for TNFRSF14 function in tumor suppression. TNFRSF14 expression could theoretically be maintained by basal p53 activity without causing cellular harm (in the absence of TNFRSF14 stimulation). In the event of malignant transformation, lymphocytes are recruited to the tumor site to eliminate tumor cells presenting tumor antigen (via MHC I and II). Since only activated T and B lymphocytes express LIGHT ligand, TNFRSF14 stimulation can serve as an additional pathway for tumor killing. Moreover, the TNFRSF14 pathway could also function as a failsafe mechanism when tumor cells evolve to escape immune surveillance (ie. downregulation of MHC molecules).

[157,158]. Under these circumstances, the presence of TNFRSF14 receptor on transformed cells could serve as a second line of defense to prevent tumor cells from evading immune surveillance. Our hypothesis here links p53 function to tumor immune surveillance, and may potentially explain why the 3KR mouse model does not develop tumor in the absence of p53-mediated apoptosis and cell cycle arrest.

It is interesting to note that TNFRSF14 can be activated by 3KR p53, but not by 4KR98, similar to the pattern observed for Mdm2 and Tigar regulation. Given the hierarchical model of p53 activation that we proposed, the p53-mediated expression of TNFRSF14 would occur under no or low stress conditions. While stimulation of TNFRSF14 leads to cell death via ligand binding, the expression of the receptor alone does not cause harm or alter cell fate. Therefore, it would be functionally appropriate if TNFRSF14 were regulated by basal p53 activity, as the process of immune surveillance would only occur in the event of malignant transformation when LIGHT-positive activated lymphocytes are present.

In summary, our studies have broadened the scope of non-canonical functions of p53. We first identified a novel p53 metabolic target, PanK1, which regulates CoA synthesis under normal physiological conditions. Even though PanK1 does not participate in p53-mediated tumor suppression, our findings shed light on the

homeostatic function of p53 and its role beyond cancer biology. We next delved into the question of how p53 differentially regulate a wide spectrum of downstream targets, especially those that carry out non-canonical p53 functions. Our data suggest that a novel p53 acetylation at K101 (K98 in mouse p53), in conjunction with other acetylation sites in the core domain, is crucial for promoter-specific activation of downstream targets. Furthermore, we characterized yet another novel non-canonical p53 target gene (TNFRSF14) that can be regulated by K101 acetylation. Efforts in elucidating the role of TNFRSF14 in tumor suppression are still underway, but our current understanding suggests that it may link p53 function to tumor immune surveillance.

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